STREPTOCOCCUS SANGUINIS ECTO-5'-NUCLEOTIDASE MODULATES PLATELET AGGREGATION

A DISSERTATION

SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL

OF THE UNIVERSITY OF MINNESOTA

BY

JINGYUAN FAN

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

MARK C. HERZBERG DDS, PhD, ADVISER

MAY 2011

ACKNOWLEDGEMENTS

I wish to express my deep gratitude here to Dr. Mark Herzberg, Dr. Gary Dunny, Dr. James White, Dr. Kylie Walters, and Dr. David Basi for serving on my dissertation committee and providing me with their precious insights for this project. I am honored to have an opportunity to work with this committee. My indebtedness is due particularly to my advisor Dr. Mark Herzberg for all of his guidance and invaluable mentoring in my four years of study in the Oral Biology PhD program at University of Minnesota and in the course of writing this dissertation. He is a role model for a great scientist, dentist and teacher whom I want to become.

Many thanks to Dr. Yongshu Zhang, Ali Khammanivong, Dr. Karen Ross, Dr. Brian Guenther, and other Herzberg lab members for their suggestion, support, and friendship. I gratefully thank Dr. Schlievert who was very patient and thoughtful in training me in doing rabbit infective endocarditis experiments. I would also acknowledge NIH/NIDCR R01DE08590 for the financial support. I also want to extend my appreciation to my former supervisor Dr. Fangchun Chen from the Department of Endodontics and Periodontics, Chongqing Stomatology Hospital, Chongqing Medical University, China for her teaching and guidance in my academic pursuit.

Finally, my thanks go to my parents and my husband, Kaibin Xu, for their many years of support. No words can express enough of my appreciation towards them.

DEDICATION

To my parents and husband for their love, understanding, encouragement and support throughout the course of thesis

ABSTRACT

Streptococcus sanguinis, an oral commensal bacterium, is the leading cause of infective endocarditis (IE). In an animal model, the abilities of *S. sanguinis* to adhere to and activate platelets are correlated with the increased severity of IE. In response to *S. sanguinis*, platelet activation is associated with secretion of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) from dense granules. The extracellular ADP is a potent platelet agonist and amplifies platelet aggregation induced by other pro-thrombotic agonists, whereas, the final product of hydrolysis of adenine nucleotides, adenosine, is a platelet aggregation antagonist.

Here, we show that cell surface ecto-5'-nucleotidase (NT5E) of *S. sanguinis* can hydrolyze adenine nucleotides ATP to ADP, adenosine monophosphate (AMP) and finally adenosine. Therefore, we hypothesize that *S. sanguinis* ecto-5'-nucleotidase modulates platelet aggregation. A *nt5e* deletion mutant of *S. sanguinis* 133-79 (Δ*nt5e*) showed significantly shorter lag time to onset of platelet aggregation than the wild-type strain (wt). However, Δ*nt5e* adhered to human platelets indistinguishably from the wild-type and complemented strains. By hydrolyzing the released ATP and ADP from dense granules of activated platelets, therefore, NT5E modulates *S. sanguinis*-induced platelet aggregation in vitro. In addition, strains of *S. sanguinis* showed different cell surface enzymatic activities for hydrolysis of adenine nucleotides, which may contribute to the

determination of the platelet interactivity phenotypes.

To further elucidate the mechanism, we distinguished the roles of ADP and adenosine receptors on streptococcal-platelet interactions using specific antagonists. We showed that the ADP receptors, $P2Y_1$ and $P2Y_{12}$, and the adenosine receptor A_{2a} were all involved in *S. sanguinis*-induced platelet aggregation. Downstream of $P2Y_{12}$, platelet activation involved two waves of Akt phosphorylation in response to *S. sanguinis*. NT5E also modulates platelet aggregation by indirectly signaling Rap1 activity. Through these pathways, *S. sanguinis* NT5E slows down platelet aggregation by removing ADP and generating adenosine.

Using a rabbit endocarditis model, we found that in the absence of *nt5e*, the mass of the vegetations and recovered bacterial loads were greatly decreased, suggesting a contribution of NT5E to the virulence of *S. sanguinis* in vivo. Similar to the release of ADP, activated platelets secrete platelet microbicidal proteins (PMPs), which antagonize a broad range of pathogens. These data, therefore, indicate that NT5E-mediated inhibition of platelet aggregation might delay presentation of PMPs to infecting bacteria on heart valves. The delay would enable the infecting bacteria to colonize in the absence of this innate immune effector. Extracellular adenine nucleotides are also important signaling molecules that mediate both inflammatory and anti-inflammatory processes. By

hydrolyzing ATP, a pro-inflammatory molecule, and generating adenosine, an immunosuppressive molecule, NT5E might inhibit phagocytic monocyte/macrophages associated with valvular vegetations, promoting the survival of infecting *S. sanguinis*.

In conclusion, we now show for the first time that streptococcal NT5E modulates *S. sanguinis*-induced platelet aggregation and contributes to the virulence of streptococci in IE. These findings expand our knowledge of bacterial-host interactions and may suggest novel therapeutics for cardiovascular infectious diseases.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

APPENDIX

CHAPTER 1

INTRODUCTION

I. The interaction of bacterial pathogens with platelets

Occasionally, opportunistic pathogens gain access into the circulatory system, resulting in a transient bacteremia. Serious vascular complications of the bacteremia, including life-threatening infective endocarditis (IE) (1), thrombocytopenia (2), and disseminated intravascular coagulation (DIC) (3), are all characterized by abnormal platelet function as a result of direct or indirect platelet-pathogen interactions.

Platelet-bacteria interactions are characterized by the binding of bacteria to platelets. The binding can be either direct (4) or indirect (5). Platelet aggregation is a crucial step in thrombus formation, which can be exploited by pathogens to cause disease. Through direct or indirect interactions, some bacterial pathogens, such as *Streptococcus sanguinis* (6) and *Staphylococcus aureus* (5), activate platelets to aggregate. When localized, such as during IE, bacterial-induced platelet aggregation results in formation of vegetations (septic thrombus) on the heart valve, which could result in heart failure (7). If the aggregation of platelets occurs in a diffuse manner, it can lead to thrombocytopenia or DIC, both of which are characterized by bleeding disorders.

Due to the increased frequency of bacteremia and antibiotic resistance, the incidence of serious cardiovascular infections has increased (1,8). Therefore, it is important to understand the mechanisms of platelet-bacteria interactions.

Biology and functions of platelets

Platelets are membrane-enclosed, cytoplasm-containing fragments of megakaryocytes. Hence, platelets are small anucleated cell-like structures that lack genomic DNA, but contain messenger RNA (9). Platelets circulate in the blood and play a fundamental role in primary hemostasis, leading to blood clotting. In diseased arteries, normal platelets may function abnormally, leading to thrombotic occlusion of the vessel, obstruction of blood flow and subsequent tissue damage (10).

Hemostasis and thrombosis. At site of vascular injury, platelet-mediated thrombus formation generally consists of three phases: initiation, propagation, and perpetuation (10,11). In the initiation phase, the endothelial cell lining of a blood vessel becomes injured or desquamated, platelets come into contact with components of the extracellular matrix, including collagen, fibrinogen and von Willebrand factor (vWF) (11). The initial adhesion of platelets at sites of injury is mediated by glycoprotein Ib/V/IX (GPIb/V/IX), a receptor complex on platelets. One component of this complex, GPIb, is the receptor for vWF. Moreover, two collagen receptors, glycoprotein VI (GPVI) and glycoprotein Ia, also have a tethering function (10). On the other hand, collagen, an insoluble platelet agonist, can directly interact with platelet receptors, GPVI or $\alpha_2\beta_1$, leading to platelet activation (11).

In the propagation phase, platelets activate, which is marked by a change in shape from resting discoid to spiky (12). Intracellular signaling events lead to rapid activation of platelet integrin αIIbβ3, the major receptor for fibrinogen, which is crucial for platelet aggregation (13) and secretion of synthesized thromboxane A_2 (TxA_2) and dense granules, which contain adenosine diphosphate (ADP), and adenosine triphosphate (ATP) (14). Secreted in response to many pro-thrombotic agonists, ADP and TxA_2 are important secondary platelet agonists, both of which propagate the aggregation cascade by activating surrounding platelets to aggregate (15). Activated integrin αIIbβ3 enables platelets to bind and become cross-linked by fibrinogen, resulting in formation of an aggregate. From the aggregate, a stable thrombus forms when the coagulation cascade enzymatically processes crosslinked fibrinogen to insoluble fibrin (16).

ADP in platelet aggregation. ADP was the first low molecular weight agonist identified for platelet aggregation (17,18). Platelets have two G protein-coupled receptors for ADP: $P2Y_1$ and $P2Y_{12}$ (19). The ADP receptor $P2Y_1$ is a G_q-coupled receptor; binding of ADP signals activation of phospholipase C (20). Signaling through $P2Y_1$ increases intracellular Ca^{2+} levels, causing shape change and transient aggregation of platelets. Another ADP receptor $P2Y_{12}$, a G_i-coupled receptor, contributes to sustained aggregation (21). The activation of $P2Y_{12}$ potentiates dense granule secretion and platelet aggregation response to other pro-thrombotic agonists such as TxA_2. Signaling through G_i, $P2Y_{12}$ inhibits

the activity of adenylate cyclase (AC), preventing formation of an internal inhibitor of platelet aggregation, cyclic AMP (cAMP). The product of AC, cAMP, is therefore reduced and activation can occur (22). The decrease in cAMP levels, however, is not directly responsible for the downstream affects of the $P2Y_{12}$ receptor (21). G_i signaling also leads to activation of PI3K, and subsequently, Akt and Rap1b. Downstream of the $P2Y_{12}$ receptor, both Akt and Rap1 are critical functional effectors.

In the bloodstream, the activation of platelets via ADP is highly regulated. In a substrate-dependent manner, ecto-adenosine triphosphate diphosphohydrolase (ecto-ATPDase, CD39), a cell-surface enzyme expressed on endothelial cells, limits the unwanted platelet aggregation by hydrolyzing ADP to adenosine monophosphate (AMP) (23). Endothelial ecto-5'-nucleotidase (CD73) can further hydrolyze AMP into phosphate and adenosine; adenosine is an aggregation antagonist (24), signaling through the A_{2a} receptor (25,26). The activation of A_{2a} by adenosine inhibits platelet aggregation through AC and leads to an increase of cAMP (27).

Upon activation, platelets also secrete ATP from dense granules. ATP and its analogues are competitive antagonists for both ADP receptors, $P2Y_1$ (28) and $P2Y_{12}$ (29). On the other hand, ATP is also involved in promoting platelet aggregation. The activation of $P2X_1$, an ATP-gated ion channel, results in fast

Ca^{2+} influx (30), which contributes to platelet activation induced by low concentrations of collagen (31). Therefore, the role of ATP in platelet aggregation is agonist dependent.

TxA_2 in platelet aggregation. When activated, platelets produce TxA_2, which amplifies aggregation by activating proximal platelets to form aggregates. Through the cyclooxygenase (COX) pathway, activated platelets synthesize TxA_2 from arachidonic acid (32). Like ADP, TxA_2 also has two G protein-coupled receptors on platelets: thromboxane prostanoid α (TPα) and thromboxane prostanoid β (TPβ) (33-35), coupled to G_1 and G_{12} or G_3, which all activate phospholipase C (36-38). Activation of TPα and TPβ leads to an increase of intracellular Ca^{2+} levels and phosphorylation of protein kinase C (39,40). However, TP receptors do not couple directly to G_i family members (25), which inhibit AC to allow platelet activation to occur (22). Platelet aggregation induced by TxA_2 also requires the secretion of ADP to inhibit AC.

Bacterial-platelet interactions

Platelets interact with bacterial pathogens through a wide array of cellular and molecular mechanisms. Generally, there are three types of bacterial-platelet interactions (41). The first is mediated by an increase in inflammatory cytokines in response to bacterial infection, which might lead to platelet activation.

Secondly, bacteria can secrete products that directly activate platelets. The third type is the binding of bacteria with platelets, which may also activate platelets to aggregate.

Bacterial-induced platelet immune response. Platelets can behave as anucleate inflammatory cells as well as hemocytes. When stimulated with microorganisms or platelet agonists associated with infection, both rabbit (42) and human platelets (43) release platelet microbicidal proteins (PMPs) and thrombin-inducible PMPs (tPMPs) in vitro. The PMPs and tPMPs can both kill (44) and exert nonlethal anti-adherence effects (45) against a wide spectrum of endovascular pathogens, including some strains of staphylococci and viridans streptococci (44). The PMPs released from platelets include platelet factor 4, connective tissue activating peptide-3 (46), RANTES (CCL5) (47), fibrinopeptide B and thymosin β (48). Pathogens resistant to PMPs and tPMPs are more virulent in vitro than their isogenic counterpart strains that are susceptible to PMPs and tPMPs (49,50).

After activation, platelets can also internalize *S. aureus* to suppress their hematogenous dissemination. However, the PMP- and tPMP-resistant *S. aureus* may survive within platelets and avoid innate immune responses (51). Depending on the strain of infecting bacteria, the outcome of platelet-bacteria interactions can differ.

Bacterial-induced platelet aggregation. A wide range of bacteria can interact with platelets (Table 1). Bacteria can adhere directly to platelets via a bacterial adhesin (4), or indirectly through plasma molecules like fibrinogen, which crosslink bacteria to platelets (5). Some bacterial pathogens can also activate platelets to aggregate by secreted bacterial products (52), direct interaction (53) or indirectly through crosslinking molecules in the plasma (5). Adhesion and activation can be either simultaneous (5) or independent processes (54) mediated by different bacterial, plasma, and platelet components. Nevertheless, bacteria can have an activating phenotype and/or an adhesive phenotype.

The interaction of platelets and bacteria can lead to platelet aggregation. Unlike the response to other agonists, platelet aggregation mediated by bacteria is all-or nothing. Aggregation, when it occurs, is always maximal and all available platelets are consumed (55). Also unlike other agonists, such as ADP and thrombin, the aggregation response to bacteria occurs after a strain and dose-dependent lag time ranging from 1.5 to 15 min (56). Why platelets aggregate after a lag time in response to bacteria is not clear. The lag time to onset of aggregation could reflect the time needed by platelets to recognize binding moieties (4,57), whereas bacteria have been suggested to only generate weak signals in platelets following initial interactions (58,59).

Table 1. Bacterial pathogens identified in IE and factors involved in bacterial-platelet interactions

Bacteria	Bacterial factor(s)	Host factor(s)	References
Streptococcus sanguinis	SrpA, PAAP	GPIb, FcγRIIa	(4,53,58,60)
Streptococcus gordonii	GspB, HsA, SspA, SspB	GPIb	(57,61,62)
Streptococcus mutans	PAc, RGP	IgG	(63,64)
Streptococcus mitis	PblA, PblB, Sm-hPAF	ND	(65)
Streptococcus agalactiae	FbsA	Fibrinogen, IgG	(66)
Streptococcus pyogenes	M protein	FcγRIIa	(67,68)
Streptococcus pneumoniae	ND	TLR2	(41)

SrpA, serine-rich protein A; PAAP, platelet-aggregation-associated protein; IgG, immunoglobulin G; PAc, protein antigen c; RGP, rhamnose-glucose polymers; ND, not determined; TLR2, Toll-like receptor 2.

Table 1. Bacterial pathogens identified in IE and factors involved in bacterial-platelet interactions (Cont.)

Bacteria	Bacterial factor(s)	Host factor(s)	References
Staphylococcus aureus	ClfA, ClfB	Fibrinogen, GPIIb/IIIa, FcγRIIa, IgG	(5,69,70)
	FnBPA, FnBPB	Fibrinogen, fibronectin, GPIIb/IIIa, FcγRIIa, IgG	
	SpA	vWF	(71)
	SdrE	Complement, IgG	(72)
	SraP, Efb	ND	(73,74)
	α-toxin	ND	(52)
Staphylococcus epidermidis	SdrG	Fibrinogen, GPIIb/IIIa, FcγRIIa, IgG	(75)
Porphyromonas gingivalis	Gingipains	FcγRIIa	(76-78)
	Hgp	GPIb	

ClfA,B, clumping factor A,B; FnBPA,B, fibronectin-binding protein A,B; SdrE,G, serine-aspartate repeat protein E,G; vWF, von Willebrand factor; Efb, extracellular fibrinogen-binding protein.

Interaction of staphylococci with platelets

In recent years, *S. aureus* is recognized as the most common cause of IE (1,79). Through several different mechanisms, *S. aureus* can bind to platelets using plasma protein linkers, and simultaneously activate platelets to aggregate (80). Generally, aggregation in response to *S. aureus* can be categorized as rapid or slow onset.

Rapid activation. When harvested from stationary phase, *S. aureus* produces clumping factor A (ClfA), which contains a fibrinogen-binding domain and plays a central role in platelet activation (69). In this model, ClfA binds fibrinogen and A domain-specific IgG. Once the two different sites on ClfA are bound, fibrinogen binds to resting αIIbβ3, which then leads to clustering of platelet Fc receptor, FcγRIIa. Clustering of FcγRIIa triggers a conformational change in integrin αIIbβ3 and platelet aggregation (81).

Exponential phase *S. aureus* express fibronectin-binding protein A (FnBPA) and fibronectin-binding protein B (FnBPB) (70). FnBPA and FnBPB have an A domain and a fibronectin binding domain. The A domain from FnBPA and FnBPB are similar to that of ClfA. In this mechanism, fibronectin bridges FnBP and the low affinity resting αIIbβ3. An αIIbβ3 activation signal is triggered when specific IgG binds to the A domain of FnBP and crosslinks to FcγRIIa.

Slow activation. *S. aureus* can activate platelets by a slower mechanism independent of αIIbβ3 (69). Deletion of the fibrinogen-binding domain in ClfA results in a prolonged lag time to onset of platelet aggregation. This activation pathway involves the specific interaction between IgG and A domain of ClfA. ClfA then binds to FcγRIIa, complement components and an unidentified platelet receptor.

Interaction of streptococci with platelets

The viridans streptococci are Gram-positive bacteria and typically commensals of the human oral cavity (82). Occasionally, viridans streptococci enter the blood stream and are among the most common causes of IE (1,8).

Streptococcus gordonii-platelet interactions. Initial studies suggested that *S. gordonii*, an oral pathogen, could only adhere to platelets and not trigger aggregation (83). However, a few strains of *S gordonii* are now recognized to use the adhesin glycoprotein GspB to bind to platelet GPIb (61,84) and activate platelets (62). Similarly, Hsa, a homologue of GspB, can bind to platelet GPIb and αIIbβ3, and plays an important role in binding to and inducing platelet aggregation (85). Since both GspB and Hsa bind, platelet receptor GPIb plays a critical role in *S. gordonii*-platelet interactions.

Moreover, *S. gordonii* also expresses high molecular weight cell-associated antigen I/II family polypeptides, SspA (172 kDa) and SspB (164 kDa) (86). Deletion of SspA and SspB from *S. gordonii* does not affect bacterial-platelet adhesion, but prolongs the lag time to platelet aggregation (57). Hence, SspA and SspB are also suggested to be involved in *S. gordonii*-induced platelet aggregation

Streptococcus sanguinis-platelet interactions. Another oral pathogen, *S. sanguinis* is one of the most common causes of IE (1,8). Our lab showed that about 60% of *S. sanguinis* strains could induce human platelets to aggregate in vitro (55,56). In an animal model, we also showed that the abilities of *S. sanguinis* to bind and activate platelets are associated with the severity of IE (87).

As we proposed, the adhesion and activation of platelets mediated by *S. sanguinis* are based on a two-site, two-step model (54). Independent of aggregation, a *S. sanguinis* adhesin, serine rich protein A (SrpA), binds to glycoprotein Ib (GPIb) on the platelet plasma membrane (60). Following adhesion, platelet activation and aggregation are triggered when the streptococcal platelet aggregation-associated protein (PAAP) binds an uncharacterized complementary signal-transducing receptor on platelets (6,53).

PAAP is a streptococcal glycoprotein and contains a collagen-like platelet-interactive domain consisting of KPGEPGPK (88). However, the interaction of bacteria with PAAP is platelet-donor specific (89).

In response to PAAP, platelets are activated and release between 0.2 and 6 µM adenosine triphosphate (ATP) and adenosine diphosphate (ADP) from their dense granules (90). ADP is a potent platelet agonist, which amplifies the aggregation response to *S. sanguinis* (91). By hydrolyzing the released ATP to ADP, the ecto-ATPase activity on *S. sanguinis* potentiates platelet aggregation in vitro (92), which could contribute to virulence in IE. However, the molecular identity of the nucleotidase activity has not been characterized.

To identify a candidate enzyme, we searched the genome of *S. sanguinis* 133-79, and a putative 5'-nucleotidase gene was found. The putative 5'-nucleotidase contains a LPXTG motif, which suggests that it anchors to the cell wall by sortase A-dependent mechanism (93). Widely distributed among bacteria, ecto-5'-nucleotidase (NT5E) is a surface-located enzyme that hydrolyzes extracellular ATP to ADP, AMP, and finally adenosine (94). Adenosine is a potent platelet antagonist (26). Since ADP and adenosine each contributes to the platelet aggregation response, we hypothesize that the 5'-nucleotidase on the surface of *S. sanguinis* modulates *S. sanguinis*-induced platelet aggregation.

II. Ecto-5'-nucleotidase: molecular structure, biochemical properties and physiological functions

5'-nucleotidase (E.C. 3.1.3.5) can catalyze the hydrolysis of the phosphoric ester bond of 5'-ribonucleotides to their corresponding ribonucleosides and phosphate. Widely distributed in bacteria, plant cells and vertebrate tissues, 5'-nucleotidase has a wide range of substrates, including 5'-purine and pyrimidine mononucleotides, 5'-dinucleotides, 5'-trinucleotides, and UDP-glucose (94). However, the major function of 5'-NT is to hydrolyze AMP to adenosine (95).

5'-nucleotidase activity is found extracellularly and in the cytosol. Cytosolic 5'-nucleotidase controls the intracellular levels of nucleoside 5'-monophosphate. In contrast, the extracellular 5'-nucleotidase (ecto-5'-nucleotidase, NT5E) is cell-surface anchored. Together with ecto-ATPDase, NT5E completely hydrolyzes extracellular ATP to adenosine and recycles extracellular nucleotides.

Molecular structure and biochemical properties

Bacterial NT5E. The bacterial NT5E (NT5E) is attached to the extracellular membrane by a glycosylphosphatidylinositol (GPI) anchor. Soluble NT5E is derived from the GPI-anchored enzyme when cleaved by endogenous phospholipases (96,97), forming non-covalently bonded homodimers (98). NT5E is highly homologous among bacteria. A NT5E from *Vibrio parahaemolyticus*

and an UDP-sugar hydrolase in *Salmonella typhimurium* have 60% and 87% identities, respectively, with the UDP-sugar hydrolase of *Escherichia coli* (94). Although the bacterial enzyme only shows about 20% identity in amino acid sequence with mammalian counterparts, there is significant sequence homology in amino acid clusters, which indicates a common ancestry (99). *E. coli* NT5E is related to mammalian NT5E and also to 2',3'-cyclic phosphodiesterases and apyrases, all of which belong to the NT5E protein family (100). Sequence motifs suggest that these enzymes belong to a larger family of metallophosphoesterases (101).

Unlike mammalian NT5E, bacterial NT5E can utilize nucleoside 5'-tri-, 5'-di-, and 5'-monophosphate (94). The K_m value for AMP is usually in the micromolar range. For enzymatic activities, divalent cations are not required, whereas Zn^{2+} is inhibitory (100).

Mammalian NT5E. The mammalian NT5E (CD73) is also tethered to the plasma membrane via a GPI-anchor. NT5E is highly expressed in endothelium, liver, and intestinal mucosa, whereas levels vary in other cells and tissues including lymphocytes, and cancer. Although it can hydrolyze a variety of nucleoside 5'-monophosphates, mammalian NT5E has the highest affinity for AMP. Together with ecto-ATPDase (CD39), NT5E completely hydrolyzes ATP to produce adenosine and phosphate (102). Adenosine 5'-[α,β-methylene] diphosphate

(APCP) is a potent inhibitor of NT5E with K_i values in the nanomolar range. In addition, ATP and ADP can also inhibit mammalian NT5E with K_i values in the micromolar range (103).

Physiological functions.

Bacterial NT5E. The native function of NT5E for the bacterial cell clearly differs from any role in platelet interactions. By catalyzing the complete hydrolysis of nucleoside triphosphates, NT5E generates phosphate and adenosine, which may be utilized by the phosphate transporter and purine salvage systems, enabling the bacterial cell to take up phosphate and adenosine needed for the metabolism.

The expression of NT5E on pathogens that invade the bloodstream has the potential to influence platelet activity. Hydrolysis of ATP and ADP may affect the activation of $P2X_1$, $P2Y_1$ and $P2Y_{12}$ receptors. NT5E can also generate adenosine, which inhibits platelet aggregation through the A_{2a} receptor. These activities contribute to the mechanism by which bacterial pathogens can interact with or even activate platelets to aggregate.

ATP and adenosine also play important roles in bacterial-host interactions. Successful host defense involves a balance of pro- and anti-inflammatory mediators. Extracellular ATP is an inflammatory signal, which induces the

release of proinflammatory cytokines such as IL-1β and IL-12 from T cells (104,105). Therefore, by hydrolyzing ATP, NT5E may inhibit ATP-induced cytokine release from host immune cells. In contrast, adenosine is a potent immunosuppressive molecule (106). Generation of adenosine by AMPases on pathogens like *S. aureus* and *Bacillus anthracis* is required for their escape from phagocytic clearance in the blood (107). Thus, NT5E could mediate immunosuppression of host cells by hydrolyzing AMP and producing adenosine. Therefore, the consumption of ATP as well as generation of adenosine by bacterial NT5E could synergize the actions of mammalian CD39 and CD73 and influence the immune response during infection.

Mammalian NT5E. The major function of mammalian NT5E (CD73) is to hydrolyze AMP to adenosine (94). Together with CD39, which cleaves extracellular ATP and ADP to AMP (108), CD73 recycles adenine nucleotides. ATP and ADP are important extracellular signaling molecules acting on P2 purinergic receptors including $P2X_1$, $P2Y_1$, and $P2Y_{12}$, whereas adenosine acts on P1 purinergic receptors such as the A_{2a} receptor. Purinergic receptor signaling is involved in a variety of biological processes including neurotransmission, platelet aggregation, and immunoregulation (109). In the vascular system, for example, endothelial cells express abundant CD73 (110), which generates adenosine from AMP. The adenosine product contributes to regulation of hemostasis through signaling A_{2a} receptor on platelets.

In mammals, adenosine assumes an essential role in regulating innate and acquired immune responses (106). Unlike other anti-inflammatory cytokines, such as IL-4, IL-10, IL-13, and TGF-β, adenosine can completely suppress immune responses (111). The immunoregulatory attributes of adenosine are mediated via four different P1 receptors: A_1, A_{2a}, A_{2b}, and A_3 (112), which are widely expressed on immune cells, such as T and B cells and neutrophils (106). Activation of A_{2a} suppresses immune responses such as effector T-cell activation and proinflammatory cytokine expression (113-115). CD73 has also been implicated in non-enzymatic functions including T cell activation and cell-cell adhesion (116,117)

In summary, NT5Es are involved in both hemostasis and host-bacterial interactions, which are crucial in the pathogenesis of cardiovascular infections. Therefore, bacterial NT5E potentially plays roles in platelet-bacterial interactions, and cardiovascular infectious diseases, such as IE.

III. Bacterial infections and infective endocarditis

Opportunistic pathogens occasionally gain access into the circulatory system, resulting in a transient bacteremia. A serious complication of the bacteremia is life-threatening infective endocarditis (IE) (83,118), which is characterized by the

formation of septic thrombi on the heart valves. IE is typically fatal if not treated with antibiotics.

Infecting microorganisms

A wide spectrum of bacterial pathogens can cause IE (80) (Table 1). However, the most frequently identified bacteria are those with highest ability to adhere to damaged heart valves. Viridans streptococci, staphylococci and enterococci, therefore, represent more than 80% of all identified pathogens from patients with IE (1). Viridans streptococci are usually commensal bacteria in the human oral cavity and cause IE in virtually all populations at risk for this infection. In contrast, staphylococcal IE was more common in elderly patients and in intravenous drug users (1,8), whereas staphylococci and enterococci were predominant pathogens identified in nosocomial endocarditis (119).

Pathogenesis of IE

During transient bacteremia, bacterial adherence to damaged heart valve tissues initiates IE (1). This event, involving both valve tissue and bacterial factors, is completed within minutes. IE also requires that bacteria persist in situ on the valve, grow within the lesions, and progressively extend to adjacent tissues. The septic “vegetative” thrombi on the heart valves can fracture and as emboli disseminate through the blood to distant organs, causing metastatic infections in the kidney, spleen and brain.

Bacterial adherence. Most patients with IE have pre-existing heart lesions (7). Any exposure of subendothelium, including extracellular matrix proteins (ECM), results in deposition of fibrin and platelets, binding of tissue factor, and formation of an aseptic thrombus on the heart valve as a normal healing process (120). This aseptic thrombus is critical for the adherence of bacteria, including streptococci and staphylococci (121,122). Adherence of bacteria to the vegetation can be mediated by interaction of bacterial adhesins, including microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), interacting with ECM from the host (123).

Infection by some bacteria, such as the viridans streptococci, can be mediated by direct interaction with platelets in the vegetation. For example, *S. sanguinis* can trigger platelet aggregation directly. PAAP, a collagen-like component of *S. sanguinis*, interacts with platelet membrane proteins of 175kDa and 230kDa to mediate platelet binding and aggregation (124). The abilities of *S. sanguinis* to adhere to and activate platelets correlate with the increased severity of IE (87).

In a third mechanism, *Staphylococcus aureus* appears to colonize heart valves without microscopically identifiable pre-existing damage (125,126). On the molecular level, however, in response to local inflammation, endothelial cells express a variety of adhesive molecules, including integrins of the β1 family

(fibronectin-binding proteins), and fibrinogen receptor αIIbβ3 (127,128). *S. aureus* expresses fibronectin-binding proteins as well as a fibrinogen-binding protein (5,69,81). To initiate infection, therefore, *S. aureus* can cross-link to superficial endothelial cells using fibronectin or fibrinogen.

Bacterial persistence. To sustain colonization and infection, bacterial pathogens must survive and persist to grow and proliferate. At this stage, a key event is maturation of vegetation, where the bacteria can become fully developed, and thus protected from host defense mechanisms (129). Local production of tissue factor (TF) and aggregation of platelets are major components of this process. Both streptococci and staphylococci can induce TF production (130) and platelet activation (87,131).

Various agonists, including cytokines and bacterial lipopolysaccharides, can trigger the production of TF. TF is a glycoprotein produced by both endothelial cells and monocytes. TF can activate factor X, leading to polymerization of fibrinogen into fibrin and also directly activate platelets, both of which are the major components of the vegetation (130). In experimental IE, TF activity in the lesions was elevated due to *Streptococcus sanguinis* and *S. aureus* (132,133). Thus, TF induction was suggested to be essential for bacterial persistence.

In comparison to TF activity, bacterial-induced platelet aggregation is a double-edged sword. On one hand, platelets aggregation contributes to the formation of vegetations (122,129). On the other hand, platelets also secrete platelet microbicidal proteins (PMPs) and thrombin-inducible PMPs (tPMPs) from α granules upon activation (43), which act against a broad spectrum of pathogens, including some strains of *S. aureus*, and viridans streptococci (42,44). However, as expected, most pathogens recovered from patients with IE were resistant to PMPs and tPMPs in vitro (49,50,134).

Bacterial invasion and dissemination. In staphylococcal IE, tissue invasion and abscess formation are prominent features during pathogenesis although prolonged untreated IE caused by most infecting bacteria can cause similar pathology. To cause invasive lesions, *S. aureus* produces a variety of virulence factors, such as exoenzymes and exotoxins (135,136). The resulting vegetative lesions are quite friable and the septic emboli can be hematogenously disseminated to organs receiving large portions of the cardiac output.

In summary, the platelet-pathogen interactions play a central role in the pathogenesis of cardiovascular infectious diseases, such as IE. As a paradigm for studies of platelet-pathogen interactions, *S. sanguinis* can adhere to and activate platelets to aggregate, both of which contribute to the severity of IE. Widely distributed among bacteria, NT5E may modulate bacterial-induced

platelet aggregation and suppress the host immune response. Therefore, *S. sanguinis* NT5E might potentially modulate platelet-bacterial interactions and contribute to the virulence of *S. sanguinis*.

CHAPTER 2

STATEMENT OF PURPOSE AND SPECIFIC AIMS

Statement of purpose

Streptococcus sanguinis is a Gram-positive oral commensal bacterium (137). Occasionally, *S. sanguinis* is inoculated into the circulating blood during dental procedures, such as tooth extraction, and cause infective endocarditis (IE) (54). In an animal model, the abilities of *S. sanguinis* to adhere to and activate platelets are associated with the severity of IE (87).

About 60% of *S. sanguinis* strains can induce human platelets to aggregate in vitro (56). In response to *S. sanguinis*, platelets are activated and release equimolar adenosine triphosphate (ATP) and adenosine diphosphate (ADP) from dense granules (90). ADP is a potent platelet agonist, which contributes to the aggregation response to many agonists, including collagen and thrombin (138). When hydrolyzed to the final product, adenosine is produced from adenine nucleotide hydrolysis. Adenosine is a platelet antagonist (26).

Widely distributed in bacteria, ecto-5'-nucleotidase (NT5E) is a surface-located enzyme that hydrolyzes 5'-nucleotides and contributes to the enzymic cascade that completely hydrolyzes extracellular ATP to adenosine (94). Thus, NT5E potentially plays a role in bacterial-induced platelet aggregation. By searching the whole genome of *S. sanguinis* 133-79, a putative *nt5e* gene was identified.

The objective of this study is to further elucidate the mechanisms of platelet-*S. sanguinis* interactions. I tested the hypothesis that *S. sanguinis* ecto-5'-nucleotidase modulates platelet aggregation. By deleting *nt5e* gene and comparing the mutant and wild-type *S. sanguinis* cells, I posited that the enzymatic activities of *S. sanguinis* NT5E could be identified and characterized. More importantly, the contribution of *S. sanguinis* NT5E to platelet aggregation could be determined. To address this hypothesis, I proposed to:

Specific aim 1: Characterize the enzymatic activities of *S. sanguinis* NT5E for the hydrolysis of adenine nucleotides.

Specific aim 2: Determine whether the cell-surface activities of NT5E contribute to the platelet-aggregation phenotypes of *S. sanguinis* strains.

Specific aim 3: Determine how *S. sanguinis* NT5E modulates platelet aggregation.

Specific aim 4: Determine whether NT5E contributes to the virulence of *S. sanguinis* in infective endocarditis.

In chapter 3, we addressed specific aims 1 and 2. We showed that *S. sanguinis* NT5E can hydrolyze ATP to ADP, AMP and finally adenosine. A *nt5e* deletion mutant of *S. sanguinis* 133-79 (Δ*nt5e*) showed significantly shorter lag time to onset of platelet aggregation than the wild-type strain (wt). However, Δ*nt5e* adhered to human platelets indistinguishably from the wild-type and

complemented strains. By hydrolyzing ATP and ADP released from dense granules of activated platelets, therefore, NT5E modulates *S. sanguinis*-induced platelet aggregation in vitro. In addition, strains of *S. sanguinis* showed different cell surface enzymatic activities for hydrolysis of adenine nucleotides, which may contribute to their platelet interactivity phenotypes.

In chapter 4, we addressed specific aim 3. Findings from chapter 3 suggested that NT5E might modulate platelet aggregation through ADP and adenosine. Using specific antagonists, we distinguished the roles of ADP and adenosine receptors on streptococcal-platelet interactions. We showed that the ADP receptors, $P2Y_1$ and $P2Y_{12}$, and the adenosine receptor A_{2a} were all involved in *S. sanguinis*-induced platelet aggregation. Downstream of $P2Y_{12}$, platelet activation involved two waves of Akt phosphorylation in response to *S. sanguinis*. NT5E also modulates platelet aggregation by indirectly signaling Rap1b activity. Through these pathways, *S. sanguinis* NT5E slows down platelet aggregation through ADP removal and adenosine generation.

In chapter 5, we reported our investigation of the role of NT5E on streptococcal IE in vivo. Using a rabbit endocarditis model, we found that in the absence of *nt5e*, the mean of vegetation masses and bacterial loads recovered from rabbits were greatly decreased, suggesting a contribution of NT5E to the virulence of *S. sanguinis* in vivo. These data indicate that NT5E promotes the survival of *S.*

sanguinis in the blood by inhibiting the secretion of platelet microbicidal proteins, and/or removing ATP and generating adenosine.

In the appendix, we investigated the role of *nt5e* in another *S. sanguinis* strain, SK36. Similarly, we found that NT5E on *S. sanguinis* SK36 also possessed ATPase, ADPase and AMPase activities. A *nt5e* deletion mutant of *S. sanguinis* SK36 showed significantly shorter lag time to onset of platelet aggregation than the wild-type strain. Like the ortholog on *S. sanguinis* 133-79, *nt5e* did not affect bacterial adhesion ability. When considered with the data in Chapter 3, the enzymatic activities of NT5E and its contribution to platelet-bacterial interactions are likely to be common among *S. sanguinis* strains.

In conclusion, we now show for the first time that streptococcal NT5E modulates *S. sanguinis*-induced platelet aggregation and may also contribute to the platelet interactivity phenotype in vitro. In addition, we also showed that NT5E contributes to the virulence of *S. sanguinis* in an animal model of IE, which may help to further elucidate the mechanisms of these infections. Thus, these findings expand our knowledge of bacterial-platelet interactions in vitro and in vivo.

CHAPTER 3

THE ECTO-5'-NUCLEOTIDASE MODULATES *STREPTOCOCCUS SANGUINIS*-INDUCED PLATELET AGGREGATION

Streptococcus sanguinis adhere to and activate human platelets to aggregate. In response to *S. sanguinis*, platelet activation is associated with secretion of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) from dense granules. The extracellular ADP is a potent platelet agonist and amplifies platelet aggregation induced by other pro-thrombotic agonists, whereas the final product of hydrolysis of adenine nucleotides, adenosine, is a platelet aggregation antagonist. Potentially modulating platelet aggregation, *S. sanguinis* cells hydrolyze extracellular ATP and ADP using uncharacterized cell wall nucleotidase activities. Here, we show that cell surface ecto-5'-nucleotidase (NT5E) of *S. sanguinis* hydrolyzes the adenine nucleotides ATP, ADP and adenosine monophosphate (AMP). A *nt5e* deletion mutant of *S. sanguinis* 133-79 (Δ*nt5e*) showed significantly shorter lag time to platelet aggregation than the wild-type strain (wt). However, Δ*nt5e* adhered to human platelets normally. By hydrolyzing the released ATP and ADP from dense granules of activated platelets, therefore, NT5E modulates *S. sanguinis*-induced platelet aggregation in vitro. In addition, strains of *S. sanguinis* showed different cell surface enzymatic activities for hydrolysis of adenine nucleotides, which may contribute to the platelet interactivity phenotypes.

INTRODUCTION

Streptococcus sanguinis is a commensal bacterium in the human oral cavity (56). When introduced into the circulation during bacteremia, *S. sanguinis* contribute to the development of infective endocarditis (139). In an animal model, the abilities of *S. sanguinis* to adhere to and activate platelets are correlated with the severity of IE (87). About 60% of *S. sanguinis* strains, however, can induce human platelets to aggregate in vitro (55,56).

S. sanguinis-induced activation and aggregation of human platelets requires several discrete interactions. Independent of aggregation, adhesion between platelets and *S. sanguinis* involves binding of an adhesin, serine rich protein A (SrpA), to glycoprotein Ib (GPIb) on the platelet plasma membrane (4,60). Following adhesion, platelet activation and aggregation are induced when the streptococcal platelet aggregation-associated protein (PAAP) binds an uncharacterized complementary signal-transducing receptor on platelets (53). In response to PAAP, platelets are activated. After activation by *S. sanguinis*, platelets first secrete between 0.2 and 6 μM adenosine triphosphate (ATP) and adenosine diphosphate (ADP) in equimolar ratios from their dense granules (90).

ADP is an aggregation agonist, which further propagates aggregation by signaling through two G protein-coupled receptors: $P2Y_1$, which increases cytosolic Ca^{2+} by activating phospholipase Cβ (PLCβ), and $P2Y_{12}$, which

suppresses cyclic AMP (cAMP) formation by inhibiting adenylate cyclase (140). When secreted in response to many agonists, dense granule stores of ADP signal to promote the platelet aggregation response (138,141). We have previously shown that ATP and ADP released from dense granules in response to *S. sanguinis* are hydrolyzed by cell wall nucleotidase activity, which appears to modulate platelet aggregation (91,92). However, the molecular identity of the nucleotidase activity has not been characterized.

Widely distributed among bacteria, ecto-5'-nucleotidase (NT5E) is a surface-located enzyme that hydrolyzes extracellular ATP to ADP, adenosine monophosphate (AMP), and adenosine (94). Adenosine is a potent platelet antagonist (26). To identify a candidate enzyme, we searched the genome of *S. sanguinis* 133-79 (wt), and a putative 5'-nucleotidase gene was found. The putative 5'-nucleotidase contains a LPXTG motif, which suggests that it anchors to the cell wall by a sortase A-dependent mechanism (93). Since ADP and adenosine each contributes to the platelet aggregation response, we hypothesize that the 5'-nucleotidase on the surface of *S. sanguinis* modulates *S. sanguinis*-induced platelet aggregation.

Strains of *S. sanguinis* display considerable variation in their ability to interact with platelets (56). Herzberg et al. identified three different streptococcal phenotypes based on their ability to bind and induce platelet aggregation.

Adh^+Agg^+ (adhesion positive and aggregation positive) can adhere to platelets and have a short lag time to platelet aggregation. $Adh^+Agg^{\pm}$ (adhesion positive and aggregation intermediate) strains can adhere to platelets and show a long lag time or delay to aggregation. Adh^-Agg^- (adhesion negative and aggregation negative) strains neither adhere nor induce platelet aggregation. Available evidence (87) suggests that the $Adh^+Agg^{\pm}$ and Adh^-Agg^- phenotypes might be less virulent as pro-thrombotic pathogens. Since adenine nucleotides are important modulators of platelet aggregation, NT5E might contribute to the platelet aggregation phenotypes of *S. sanguinis* strains. Therefore, we also examined whole cell enzymatic activities on strains with different aggregation phenotypes.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. sanguinis* strains were routinely grown in Todd Hewitt broth (TH broth, Difco; Sparks, MD) or on TH agar plates at 37℃ in 5% CO_2. *E. coli* cells were grown aerobically at 37℃ in Luria-Bertani broth (LB broth, Bacto; Sparks, MD). When required, antibiotics were added to the medium at the indicated concentrations: erythromycin (Em), 10 µg ml^{-1} (*S. sanguinis*); and kanamycin (Km), 50 µg ml^{-1} (*E. coli*) or 400 µg ml^{-1} (*S. sanguinis*).

Genetic manipulations in *S. sanguinis* 133-79. Standard recombinant DNA techniques were employed as described (142). Plasmids (listed in Table 1) were

purified from *E. coli* cells using the QIAquick Spin Miniprep purification Kit (Qiagen Inc., Valencia, CA). Oligonucleotides (Table 2) were synthesized using integrated DNA Technologies.

Chromosomal DNA was prepared from mutanolysin-treated streptococcal cells using the Qiagen 100/G Genomic Tip system (143). PCR products were purified using the High Pure PCR Product Purification Kit (Roche Ltd., Indianapolis, IN). DNA restriction and modification enzymes were used according to the manufacturer's directions (New England Biolabs Inc., Ipswich, MA).

The *nt5e* operon of *S. sanguinis* 133-79 (wt) was inactivated by allelic exchange with the erythromycin-resistance determinant, *ermAM*. Briefly, *ermAM* was amplified from plasmid pVA891 (144) and cloned into pPCR-Amp SK (+) (Stratagene Corp., La Jolla, CA). Two DNA fragments constituting the flanking sequences of the *nt5e* operon were then amplified and fused with the *ermAM* genes sequentially (145). The fused construct was then PCR-amplified, purified and transformed into *S. sanguinis* 133-79. For transformation, overnight cultures were grown in TH broth. The next day, cells were freshly inoculated in the same medium (1:40), containing 10% heat-inactivated horse serum (Sigma-Aldrich, St. Louis, MO), and the plasmid carrying the erythromycin-resistant determinant was added. Competence stimulating peptide (CSP, a gift from Dr. Jens Kreth, University of Oklahoma) was added to a final concentration of 200 ng ml^{-1}.

Incubation continued for 5h at 37ºC and cells were plated on Em selective TH plates, generating the NT5E deletion mutant (Δ*nt5e*). The insertions were confirmed by PCR amplification and sequencing.

To complement *Δnt5e*, a DNA fragment encompassing the entire *nt5e* operon was amplified by PCR from wt and cloned into the *E. coli*-streptococcal shuttle vector pDL276 (146), generating plasmid pDL276-*nt5e*. The construct was amplified in *E. coli*, purified and used to transform the Δ*nt5e* to obtain the complemented strain *nt5e*+ using the method described above. Predicted insertions were confirmed by PCR amplification and sequencing.

Nucleotidase activity.

AMPase activity. Streptococcal cells in stationary phase were harvested after 16 h in THB. Harvested cells were washed twice with 30 mM Tris•HCl buffer (pH 7.4) containing 0.25 mM ethylenediamine tetraacetic acid (EDTA) and 30 mM sodium chloride (NaCl), followed by washing with 50 mM Tris•HCl (pH 7.4) containing 130 mM sodium chloride (NaCl) and 5 mM magnesium dichloride ($MgCl_2$), and resuspended to 2 X 10^9 cells per ml. To each 2 ml microcentrifuge tube, 0.5 ml bacterial suspension was mixed with AMP added to a final concentration of 5 to 100 µM and incubated at 37℃ for 30 minutes. After incubation, cells were centrifuged at 10,000 x g for 5min, and 50 µl of the supernatant from each tube was transferred into a 96-well plates (Corning, NY).

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
E. coli strain DH5α F'I^q	F' *proA*$^+$*B*$^+$ *lacI*^{q} Δ*(lacZ)M15 zzf*::Tn10(Tet^R)/fhuA2Δ(*argF-lacZ)U169 phoA glnV44 φ80* Δ*(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England Biolabs
S. sanguinis strains		
133-79	Wild-type	(56)
133-79Δ*nt5e*	Δ(*nt5e*) : : *ermAM* (Em^R)	This study
133-79*nt5e*+	Δ(*nt5e*) : : *ermAM* (pDL276 *nt5e*) (Em^R,Km^R)	This study
Plasmids		
pPCR-Amp SK (+)	3.0 kb; Ap^R; pUC*ori*	Stratagene
pDL276	6.9 kb; Km^R; ColE1*ori*, *E. coli*–streptococcal shuttle vector	(146)
pVA891	5.4 kb; Em^R, Cm^R; pACYC*ori*, *E. coli*–streptococcal shuttle vector	(144)

Table 2. Primers used in this study

Primer	Sequence (5’ to 3’)
133-79A1F	ACGTCCGGTACCAGCAAAGGCCACATCCATAG
133-79A1R	ACGTCCGAATTCCGGTGGTTACCTGCAAATCT
133-79A2F	ACGTCCGGATCCATGGTTTCCGACTGCCATAG
133-79A2R	ACGTCCGAGCTCAAAAGGCGTTATCGGTGATG
133-79ACom_F	ACGTCCGTCGACCTCTGATGAATTTCAGAGTC
133-79ACom_R	ACGTCCGAGCTCCTAGTCTTCTTTATGACTTT

[a] All primers were designed as part of this study.

[b] Underlined letters indicate restriction enzyme site.

Similarly, the crude tryptic digest or fractions from gel filtration (final concentration 10 μg/ml) were incubated with 50 μM AMP at 37℃ for 15 minutes. The reactions were stopped by equal volume of HCl (final concentration 0.1N) and 50 μl of the final was transferred into a 96 well plates as well. The AMPase activity was measured as the amount of inorganic phosphate (Pi) released into the supernatants using the QuantiChrom Phosphate Assay Kit DIPI-500 (Bioassay systems, Hayward, CA). The results were expressed as nM of Pi produced/min per 10^6 cells or nM of Pi produced/min per ng of protein. The K_m (Michaelis constant) and V_{max} (maximum velocity) for AMPase activity of intact *S. sanguinis* cells were also determined. To minimize the likelihood that Pi was generated by other enzymes, some streptococcal cells were pretreated with 1 μM to 1 mM tetramisole (Sigma-Aldrich), an inhibitor of alkaline phosphatase, or 1 μM to 1 mM adenosine 5'-[α,β-methylene] diphosphate (APCP) (Sigma-Aldrich), an inhibitor of NT5E, before incubation with AMP.

Phosphate hydrolase activity. After removal of the culture medium, the streptococcal cells were washed twice with 50 mM Tris•HCl (pH 8.0) buffer containing 150 mM NaCl, 5 mM $CaCl_2$, and 5 mM $MgCl_2$. The cells were then incubated with 50 to 400 μl ADP or ATP at 37℃ for 30 minutes. To quantify the enzymatic activities, the crude tryptic digest or fractions from gel filtration (final concentration 10 μg/ml) were incubated with 50 μM ADP or ATP at 37℃ for 30 minutes. The reactions were stopped by equal volume of HCl (final

concentration 0.1 N). The enzymatic activity was measured as described above.

Biochemical characterization of NT5E in cell wall fractions. Minimal tryptic digests of *S. sanguinis* 133-79 were prepared as described (53), which leaves the cell wall intact. The crude digests representing minimally digested surface proteins were concentrated and desalted using an ultrafiltration column (10 kDa cutoff; Millipore, Billerica, MA) into 2 mL distilled water (dH_2O). The salt-free protein fragment concentrates were then chromatographed on a column (1.25 X 95 cm) of Sephadex G-100 (GE Health) at a flow rate of 0.3 ml/min in PBS. The fraction with the greatest ability to inhibit *S. sanguinis*-induced platelet rich plasma (PRP) aggregation (G100-3) was then separated using two-dimensional SDS gel electrophoresis. Gels were stained with silver stain and spots were excised for mass spectrometry analysis (Center for Mass Spectrometry and Proteomics, University of Minnesota).

.

Platelet aggregometry. Strains of *S. sanguinis* were tested for the ability to induce platelet aggregation with fresh PRP obtained from a single donor as described previously (55). A single donor was used to eliminate variability in platelet response between donors (139). Each bacterial strain (50 µl suspension containing 4 X 10^9 cells/ml) was incubated with 450 µl of PRP (4 X 10^8 cells/ml). PRP aggregation was performed at 37℃ with controlled stirring in a recording

aggregometer (model 660, Chronolog Corp., Havertown, PA), and the lag time or delay to onset (minutes) was measured.

Platelet bacterial adhesion assay (PBAA). All procedures were performed as described previously (55). In brief, platelets from outdated PRP (Memorial Blood Center, St. Paul, MN) were washed with PBS and fixed with 10% formalin. Washed platelets and washed streptococcal cells were incubated together or alone (controls) in microwells; the small clusters of adhering platelets and bacteria were separated from non-interacting particles by centrifugation. The sedimentation of adhering mixtures relative to controls was quantitated by the following formula: percent adhesion = 100 X {1 – [mixture $A_{620\ nm}$/(bacterium $A_{620\ nm}$ + washed-platelet $A_{620\ nm}$)/2]}. Based on previous studies of the variability of the method, only adhesion scores of ≥ 20% were considered positive.

Gene sequences. We have partially sequenced the genome of *S. sanguinis* 133-79. The putative *nt5e* gene of *S. sanguinis* 133-79 shared a 95% sequence identity with that of *S. sanguinis* SK36 (147).

Statistical analysis. Descriptive statistics, including means and standard errors, were calculated. Statistical analysis of data was performed using the Student's *t*-test, one-way analysis of variance (ANOVA), non-linear regression or 4-parameter logistic regression (4-PL) with GraphPad Prism 5 (GraphPad

Software, La Jolla, CA). The level of significance $\alpha = 0.05$ was considered to be statistically significant.

RESULTS

Characterization of the enzymatic activities of *S. sanguinis* NT5E. We measured the *S. sanguinis* per cell NT5E activity. Following Michaelis-Menten kinetics, *S. sanguinis* wild-type (wt) hydrolyzed the adenine nucleotides ATP (Figure 1A), ADP (Figure 1B) and AMP (Figure 1C). For wt strain ATP hydrolysis, K_m was 118.0 $\pm$ 35.1 µM and V_{max} was 9.0 $\pm$ 1.0 nmoles Pi/min per 10^6 cells. Using the Δ*nt5e* strain (K_m = 396.0 $\pm$ 25.9 µM, V_{max} = 1.7 $\pm$ 0.9 nmoles Pi/min per 10^6 cells), the ATPase activity was significantly decreased ($P < 0.01$) whereas activity in this mutant was restored to wt levels by complementation (*nt5e*+) (Figure 1A) (K_m = 124.7 $\pm$ 33.8 µM, V_{max} = 8.3 $\pm$ 0.9 nmoles Pi/min per 10^6 cells). For wt strain ADP hydrolysis, K_m was 65.7 $\pm$ 20.0 µM and V_{max} was 5.4 $\pm$ 0.5 nmoles Pi/min per 10^6 cells. In the absence of *nt5e*, the ADPase activity was ablated whereas activity in this mutant was restored to wt levels by complementation (*nt5e*+) (Figure 1B) (K_m = 81.7 $\pm$ 21.1 µM, V_{max} = 5.4 $\pm$ 0.5 nmoles Pi/min per 10^6 cells). Similarly, AMPase activity in *S. sanguinis* 133-79 (K_m = 38.9 $\pm$ 15.6 µM, V_{max} = 1.0 $\pm$ 0.2 nmoles Pi/min per 10^6 cells) was fully abrogated by deletion of *nt5e* (Figure 1C). When complemented, *nt5e*+ regained AMPase activity (K_m = 40.0 $\pm$ 7.2 µM, V_{max} = 0.9 $\pm$ 0.1 nmoles Pi/min per 10^6

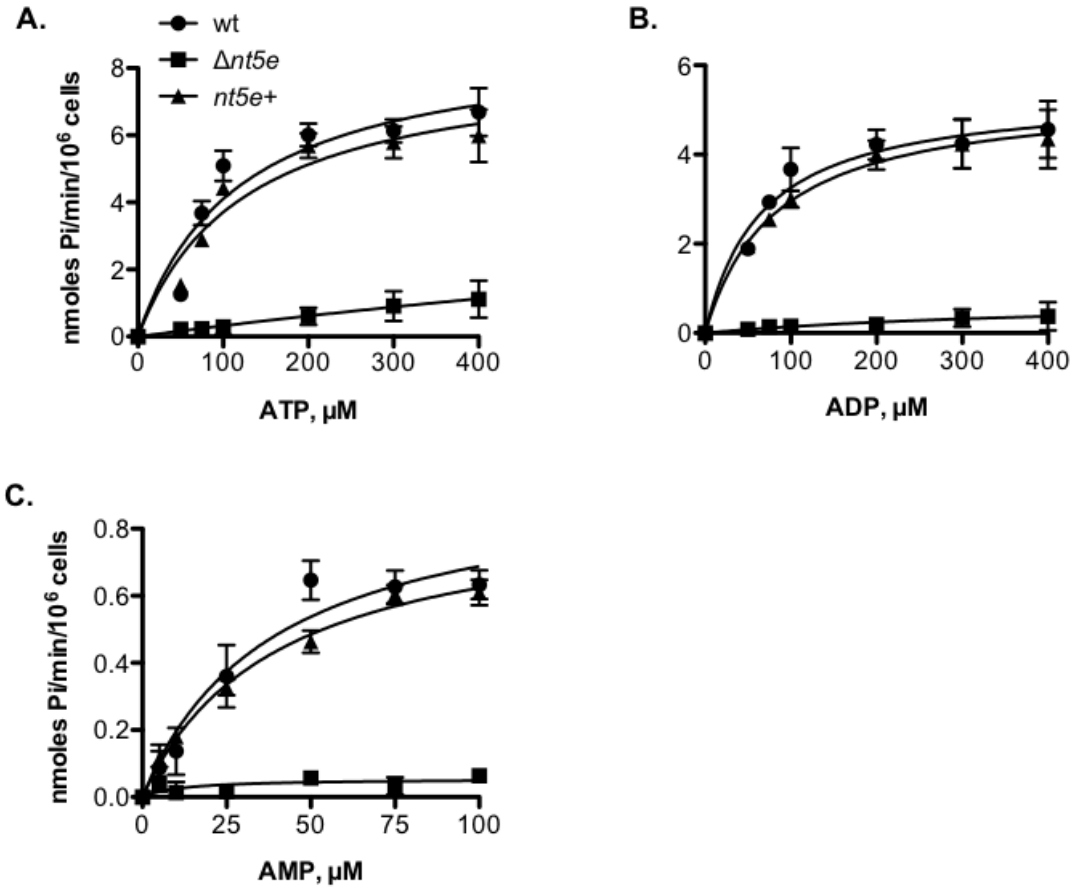


Figure 1. Characterization of NT5E activity on *S. sanguinis* 133-79 whole cell.

NT5E activity was measured by the release of inorganic phosphate (Pi) from adenine nucleotides. For (A), (B), and (C), the Michaelis-Menten curves were showed as enzyme velocity vs. concentration of ATP, ADP and AMP substrates. The results were represented as Mean ± SE, n = 3.

cells). Indeed, only with ATP as substrate, Δ*nt5e* still generated inorganic phosphate. This finding was consistent with the concomitant presence of an ecto-ATPase on the cell surface of *S. sanguinis* as we reported (92). Furthermore, compared to NT5E, this ecto-ATPase did not play a major role in the hydrolysis of extracellular adenine nucleotides. Collectively, these results suggest strongly that NT5E on *S. sanguinis* 133-79 hydrolyzes the adenine nucleotides ATP, ADP and AMP.

AMPase activity attributed to NT5E. Since our assays were performed with whole cells, we considered the possibility that other phosphatases could contribute to the total enzyme activity. A search of the *S. sanguinis* genome suggested the possible expression of two putative alkaline phosphatase (AP) ectoenzymes (147). Unlike NT5E, AP is sensitive to an alkaline pH optimum (148). The AMPase activity did not increase with increasing pH (Figure 2C). However, the AMPase activity was inhibited by α,β-methylene ADP (APCP), a known inhibitor of NT5E (Figure 2A and 2B). When concentration was expressed on a logarithmic scale, the APCP curve was fitted to a sigmoidal, single-site model (Figure 2A), allowing determination of the half-maximal inhibitory concentration (IC_{50} = 8.8 $\pm$ 3.8 μM). In the presence of APCP (K_m = 66.7 $\pm$ 14.1 μM; V_{max} = 0.9 $\pm$ 0.1 nmoles Pi/min per 10^6 cells), the whole cell AMPase activity showed an apparent increase in K_m ($P < 0.05$) but no increase in the V_{max} ($P = 0.3$) compared to no inhibitor control (K_m = 38.9 $\pm$ 15.6 μM; V_{max} =

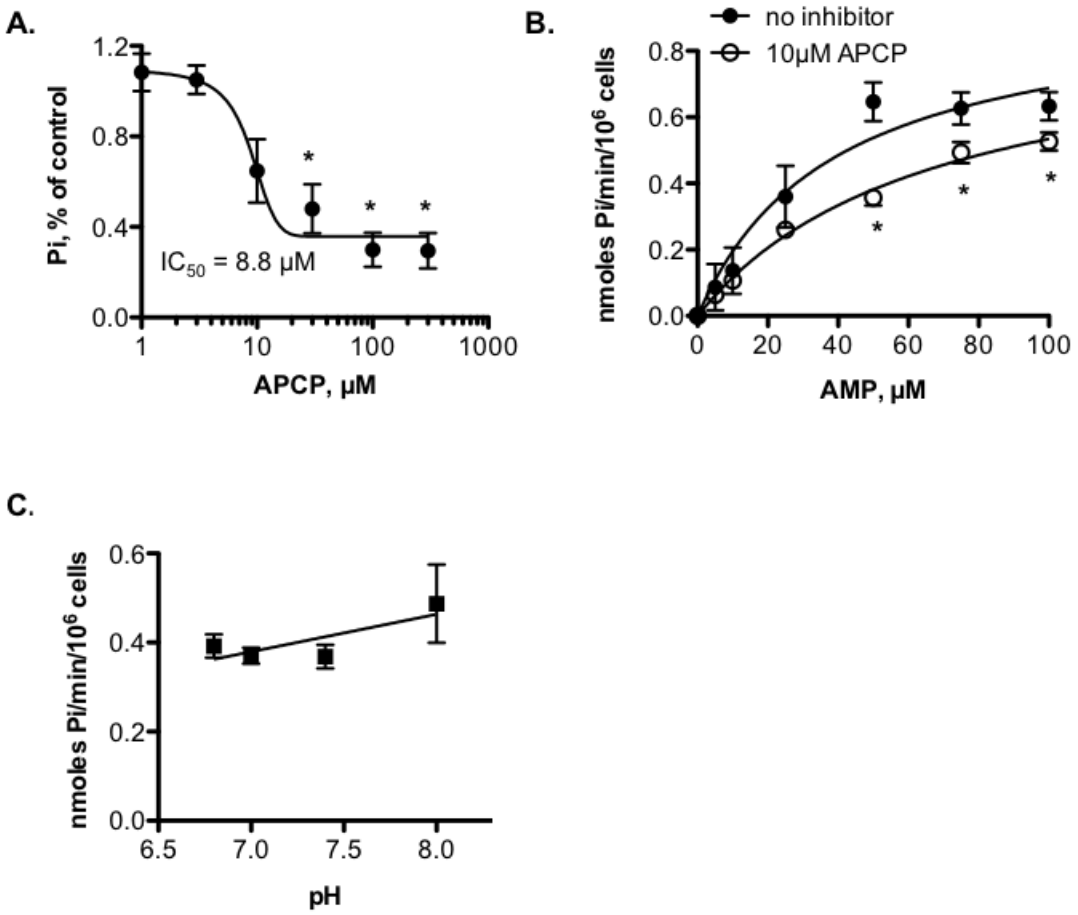


Figure 2. The AMPase activity on *S. sanguinis* 133-79 whole cell is attributed to NT5E rather than AP.

(A) effect of NT5E inhibitor APCP on enzymatic activity of *S. sanguinis* 133-79. The curve was fitted to a sigmoidal inhibitory dose-response curve and the inhibitory concentration 50% (IC_{50}) value derived from the curve fit was shown. (B) Michealis-Menten curves of enzyme activity vs. substrate concentration in the absence and presence of APCP. (C) pH dependence of NT5E activity. The results were represented as Mean ± SE, n = 3; * significantly decreased compared to no inhibitor ($P < 0.05$).

1.0 $\pm$ 0.2 nmoles Pi/min per 10^6 cells). Since the inhibition could be overcome at high concentrations of AMP substrate, APCP competitively inhibited AMPase activity (Figure 2B). Therefore, the AMPase activity was not AP but NT5E.

Identification of NT5E as a trypsin-resistant protein from cell surface of *S. sanguinis.* Recovery of the cell surface proteins fragments from *S. sanguinis* 133-79 was maximal after 7 min of TPCK-trypsin digestion (data not shown). The 7-min tryptic digests (crude digest) of *S. sanguinis* 133-79 were chromatographed on a column of Sephadex G-100, pooled (Figure 3A and 3B), and analyzed for platelet interactions (Table 3). Fraction 3 (G100-3) had the greatest ability to inhibit *S. sanguinis*-induced platelet aggregation (Table 3) but had no effect on platelet-*S. sanguinis* adhesion (data not shown). After separation on two-dimensional SDS gel electrophoresis and analysis by mass spectrometry, two putative 5'-nucleotidase superfamily proteins were identified (147) (Table 4). The crude digest and fraction G100-3 showed 5'-nucleotidase activities (Table 3). Furthermore, compared with the crude digest, G100-3 had higher AMPase, ADPase as well as ATPase activity, which reconciled with the inhibition of platelet aggregation (Table 3). In the plasma, both ADP removal and adenosine generation can inhibit platelet aggregation. Taken together, 5'-nucleotidase was a tryptic-resistant protein on the surface of *S. sanguinis* 133-79 and potentially modulated platelet aggregation by metabolizing adenine nucleotides.

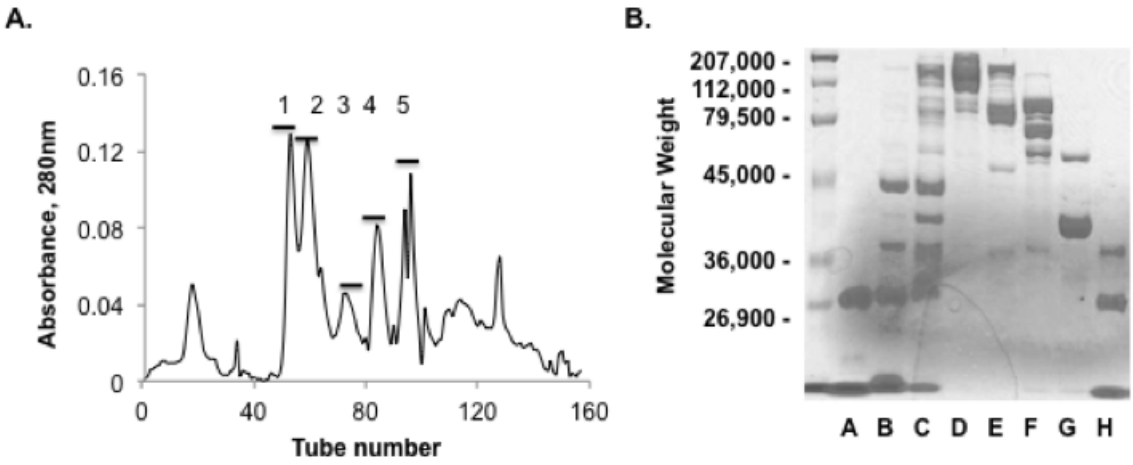


Figure 3. Gel filtration chromatography of trypsin-sensitive proteins from whole cells of *S. sanguinis.*

(A) Gel filtration chromatography of 7 minutes tryptic digest of *S. sanguinis* 133-79. 6.4 mg was placed on a column of Sephadex G-100 and chromatographed as described under “Materials and methods”. (B) SDS-PAGE analysis of *S. sanguinis* tryptic digest fractions from gel filtration chromatography. All samples contained 15 µg of protein solubilized in 1% (w/v) SDS sample buffer. These samples were electrophoresed on a 10% gel, and stained with Coomassie Blue. Lane A, trypsin. Lane B, flow-through from void volume. Lane C, starting 7-min crude tryptic digest. Lane D, Sephadex G-100 fraction 1. Lane E, Sephadex G-100 fraction 2. Lane F, Sephadex G-100 fraction 3. Lane G, Sephadex G-100 fraction 4. Lane H, Sephadex G-100 fraction 5.

Table 3. Recovery of Sephadex G100 fractions of *S. sanguinis* tryptic digest

Fraction	Protein, mg recovered (%)	PRP aggregation onset min[a]	Hydrolysis of nmoles Pi/ng/min[b]		
			AMP	ADP	ATP
none	-	7.5	-	-	-
crude digest	6.4	9.8	0.15	0.12	0.11
fraction 1	1.3 (20.3)	7.5	0.01	0.01	0.01
2	1.7 (26.6)	7.8	0.12	0.10	0.09
3	1.0 (15.6)	> 30	0.59	0.55	0.42
4	0.8 (12.5)	7.6	0.01	0.01	0
5	0.8 (12.5)	7.5	0.02	0	0
recovered total	5.6 (87.5)				

[a] PRP was preincubated with the indicated fraction at a final concentration of 0.1mg/ml.

[b] Proteins were incubated with 50 µM of AMP, ADP or ATP for 15min at 37 ℃ at a final concentration of 10 µg/ml.

Table 4. Proteins in G100-3 identified by mass spectrometry

Identified proteins	Accession No.	M.W. (KDa)	Substrate specificity
5'-nucleotidase, putative (*S. sanguinis* SK36)	gi|125718054	76	5’-nucleotides with preference for adenine nucleotides
cyclo-nucleotide phosphodiesterase (*S. sanguinis* SK36)	gi|125717119	87	Nucleoside 2',3'-cyclic phosphate into nucleoside 3'-phosphate
cyclo-nucleotide phosphodiesterase (*S. suis* 98HAH33)	gi|146321945	88	Nucleoside 2',3'-cyclic phosphate into nucleoside 3'-phosphate
5'-nucleotidase family protein (*S. gordonii* CH1)	gi|157150885	78	5’-nucleotides with preference for adenine nucleotides

*M. W. = molecular weight, *S. suis* = *Streptococcus suis*, and *S. gordonii* = *Streptococcus gordonii*.

Inhibition of platelet aggregation by NT5E. Using platelet aggregometry, we compared the aggregation lag-time and magnitude in response to wt, Δ*nt5e* and *nt5e*+. In response to either wt or *nt5e*+, platelets from a single donor aggregated in approximately 9 min (Figure 4A). The aggregation response of Δ*nt5e* showed a lag-time of approximately 5 min, which was significantly shorter than the wt and *nt5e*+ strains (Figure 4B). The magnitude of aggregation, however, was similar in response to all three strains (Figure 4A). Among three donors we tested, the differences in aggregation response to wt, Δ*nt5e* and *nt5e*+ were consistent (data not shown). Therefore, the *S. sanguinis* NT5E prolonged the lag time to the onset of platelet aggregation.

Adhesion of platelets and bacteria. The percent adhesion to platelets was similar for wt (60.1 ± 0.9%), Δ*nt5e* (59.9 ± 1.2%) and *nt5e*+ (59.6 ± 2.6%) strains.

NT5E contributes to the platelet interactivity phenotype. Several *S. sanguinis* strains were characterized for whole cell hydrolysis of adenine nucleotides (Table 5). Differing from other bacteria that hydrolyze AMP, ADP and ATP with comparable K_m (149,150), *S. sanguinis* strains preferentially hydrolyzed AMP (lower K_m) rather than ADP and ATP (Table 5). Interestingly, NT5E activity also differed among strains with different aggregation phenotypes. For example, K_m for AMPase activity of *S. sanguinis* 133-79, an Adh+Agg+ strain, was significantly lower than that of L22, an Adh+Agg± strain ($P < 0.01$).

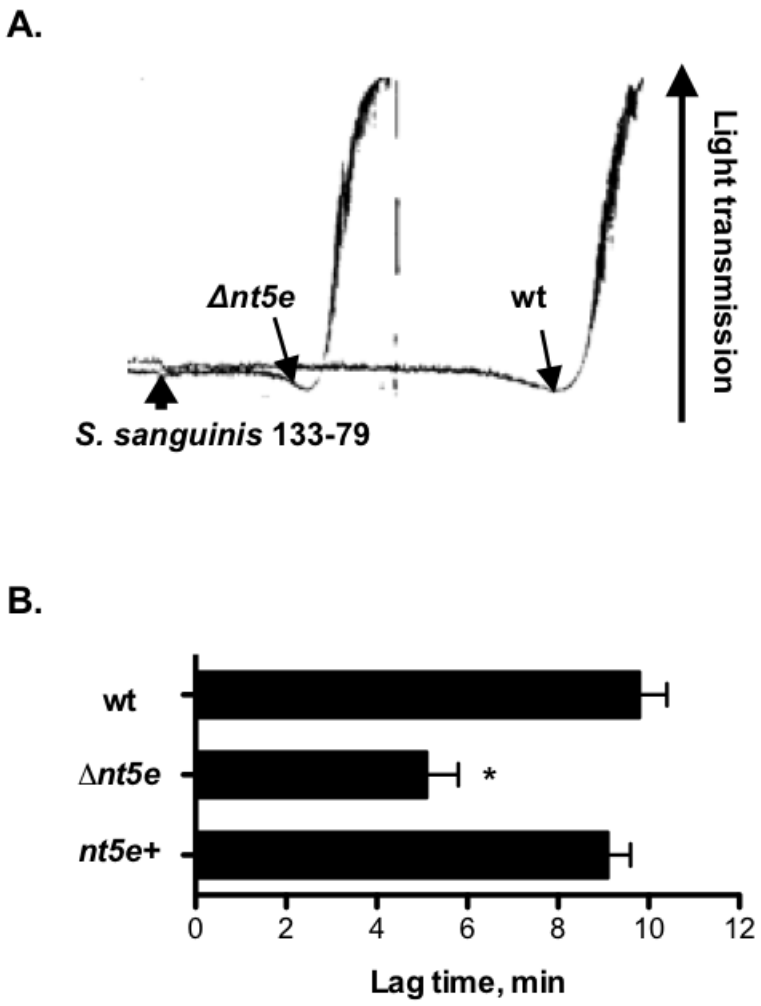


Figure 4. NT5E affects platelet aggregation lag time.

(A) PRP was stirred in an aggregometer. *S. sanguinis* strains was added as pointed by the arrowhead and aggregation measured as increasing light. The start of aggregation was indicated by arrow. (B) Response leading to aggregation was recorded as the mean lag-time to onset of aggregation ± SE, N=4; * significantly decresed compared to wt ($P < 0.05$).

Table 5. Characterization of the enzymatic activities of *S. sanguinis* strains

Strain	Adhesion (%)	PRP aggregation onset (min)	Phenotype[a]	Hydrolysis of (Km, µM)[b]		
				Phosphate Hydrolase		
				AMP	ADP	ATP
133-79	58	7.8	Adh+ Agg+	38.9 ± 15.6	65.7 ± 20.0	118.0 ± 35.1
L52	48	8.9	Adh+ Agg+	41.3 ± 13.2	87.2 ± 24.1	102.6 ± 18.4
L14	53	12.7	Adh+ Agg±	31.7 ± 3.4	68.8 ± 16.2	80.2 ± 27.1
10558	78	14	Adh+ Agg±	78.5 ± 12.4	122.8 ± 38.2	134.5 ± 25.0
L22	52	12.4	Adh+ Agg±	73.3 ± 18.7	97.2 ± 20.0	102.3 ± 28.8
L13	36	> 20	Adh+ Agg-	101.2 ± 18.0	-	-
L74	42	> 20	Adh+ Agg-	187.0 ± 25.5	-	-

[a] Phenotype and % adhesion or time to the onset of aggregation: Adh-, < 10%; Adh±, 10 to 24%; Adh+, ≥ 25%; Agg-, > 20 min; Agg±, 10 to 20 min; Agg+, < 10 min.

[b] Whole bacterial cell surface enzymatic activities

As reported, the AMPase, ADPase and part of ATPase activities are almost all attributed to NT5E on *S. sanguinis* 133-79. Therefore, the nucleotidase activities of NT5E may contribute to the platelet interactivity phenotype of strains of *S. sanguinis*.

DISCUSSION

We have previously detected nucleotidase activities on the cell wall of *S. sanguinis* (91,92). The ecto-nucleotidase activity could hydrolyze ATP and ADP released from dense granules and modulate platelet aggregation in vitro. As we sought to further identify and characterize surface nucleotidase activities, we identified an NT5E on *S. sanguinis* 133-79. This NT5E can hydrolyze extracellular ATP to ADP, AMP and adenosine. Since ADP and adenosine each contributes to the platelet aggregation response, we hypothesized that the *S. sanguinis* NT5E modulates platelet aggregation.

The aggregation response to bacteria differs from other agonists, such as thrombin and collagen. The response to most species of bacteria is all-or-none and the strength of the response is recognized by lag times to onset of aggregation that are increasingly short (55,151). The lag time to onset of platelet aggregation is also a characteristic of bacterial strain-human platelet donor pair. When induced by the strains of *S. sanguinis* we have studied, platelet aggregation in vitro occurs after a lag time of 1.5 to 15 min depending on the

platelet donor and the strain of *S. sanguinis* (55,56). We now show that the lag time is in part a response to the NT5E activity on the surface of cells of many species.

An *nt5e* deletion mutant of *S. sanguinis* 133-79 showed a shorter platelet aggregation lag time but similar magnitude of aggregation when compared to the parental strain (Figure 4). Inactivation of *nt5e* gene, however, did not affect the ability of *S. sanguinis* 133-79 to adhere to human platelets. Since *S. sanguinis*-induced activation and aggregation of human platelets requires adhesion and aggregation, these results suggested that NT5E modulated the aggregation lag-time independently of adhesion.

NT5E was released from the cell surface by trypsin digestion. When the digest was fractionated using gel filtration chromatography, NT5E was identified in one of the fractions, G100-3 (Table 4). In the presence of ATP and ADP secreted from activated platelets, the NT5E activity can hydrolyze ADP, an agonist of platelet aggregation, and generate adenosine, an inhibitor of platelet aggregation. It was not surprising, therefore, that fraction G100-3 inhibited platelet aggregation (Table 3). When compared to the crude digest, G100-3 was enriched for NT5E activity towards nucleotide substrates. Since the NT5E activity was definitively shown to be present in G100-3, the data show that this enzyme is an ecto-enzyme that can remove platelet agonist ADP and produce

aggregation inhibitor adenosine during *S. sanguinis* interactions with platelets.

To determine whether NT5E activity distinguishes the platelet-aggregation phenotypes of *S. sanguinis* strains, we compared whole cell adenine nucleotides hydrolysis for several *S. sanguinis* strains. All strains of *S. sanguinis* characterized had higher preference for hydrolysis of AMP than ADP and ATP (Table 5). Interestingly, the activities on strains with different aggregation phenotypes were dramatically different. Generally, the enzymatic activities on Adh+Agg+ strains were the highest among three different platelet interactivity phenotypes, suggesting that NT5E contributes to the bacterial-platelet interactivity phenotype in concert with other factors such as adhesin and PAAP expression. To better define the role of NT5E, more *S. sanguinis* strains need to be characterized and multiple variables must be included into the regression analysis.

In conclusion, we provide evidence that NT5E is a modulator of *S. sanguinis*-induced platelet aggregation in vitro. Studies are on going to elucidate the potential signaling pathway in platelets induced by *S. sanguinis*. These data will provide valuable information for understanding the mechanism of bacteria-induced platelet aggregation in septic thrombus formation.

CHAPTER 4

STREPTOCOCCUS SANGUINIS ECTO-5'-NUCLEOTIDASE MODULATES PLATELET AGGREGATION THROUGH P2 RECEPTORS AND A_{2A} RECEPTOR

In a rabbit model, the ability of *Streptococcus sanguinis* to induce platelets to aggregate is associated with the severity of infective endocarditis. Upon activation by *S. sanguinis*, platelets secrete dense granules, which contain equimolar ATP and ADP. An ecto-5'-nucleotidase (NT5E) on *S. sanguinis* 133-79 has been shown to prolong the lag time to the onset of platelet aggregation in vitro. By hydrolyzing extracellular adenine nucleotides, NT5E potentially modulates platelet aggregation through ADP, the major agonist for platelet aggregation, and adenosine, an inhibitor of platelet aggregation. In response to ADP, platelet activation is mediated via $P2Y_1$ and $P2Y_{12}$ receptors. In a dose-dependent manner, antagonists of $P2Y_1$ and $P2Y_{12}$ receptors prolonged lag times to platelet aggregation mediated by both wild-type *S. sanguinis* 133-79 (wt) and the isogenic NT5E deletion mutant (Δ*nt5e*). However, the wt strain is more sensitive to the antagonists than Δ*nt5e*, reflecting an ADP-dependent response. In contrast, an antagonist of adenosine A_{2a} receptor only accelerated platelet aggregation mediated by the wt strain where NT5E was present. Downstream of the $P2Y_{12}$ receptor, *S. sanguinis* induced-platelet activation involves two waves of Akt phosphorylation and activation of Rap1b. However, in response to wt, Rap1b was de-activated and then reactivated during the lag phase before aggregation, whereas, Rap1b was continually active in response to Δ*nt5e*. Therefore, *S. sanguinis* NT5E modulates platelet aggregation through ADP receptors, $P2Y_1$ and $P2Y_{12}$, and adenosine receptor A_{2a}.

INTRODUCTION

Streptococcus sanguinis, a Gram-positive oral bacterium, is one of the most common pathogens isolated from infective endocarditis (IE) patients (1,8). Studies using an animal model have shown that the abilities of *S. sanguinis* to adhere to and activate platelets are associated with the severity of IE (87).

Upon activation by *S. sanguinis*, platelets secrete 0.2 to 6 μM adenine triphosphate (ATP) and adenine diphosphate (ADP) in equimolar ratios (90). The extracellular ADP is a potent platelet agonist and amplifies platelet aggregation induced by other pro-thrombotic agonists (140), whereas, the final product of hydrolysis of adenine nucleotides, adenosine, is a platelet antagonist, which inhibits platelet aggregation (26). When expressed on the surface of bacteria that invade the bloodstream, ecto-nucleotidases, which hydrolyze adenine nucleotides, have the potential to influence platelet activity (26).

We have identified and characterized an ecto-5'-nucleotidase (NT5E) on *S. sanguinis* 133-79 (chapter 3). The NT5E on the surface of *S. sanguinis* 133-79 can hydrolyze the extracellular adenine nucleotides ATP, ADP, adenosine monophosphate (AMP) to adenosine. Deletion of NT5E did not affect the magnitude of *S. sanguinis*-platelet adhesion or aggregation, but the lag time to platelet aggregation decreased. Therefore, NT5E potentially modulates *S. sanguinis*-induced platelet aggregation by metabolizing adenine nucleotides ATP

and ADP from dense granules. However, the platelet response mechanisms underlying modulation have not been characterized.

Human platelets have three types of P2 receptors: the G protein-coupled P2Y receptors, $P2Y_1$ and $P2Y_{12}$, which are both activated by ADP (140), and an ion channel $P2X_1$ receptor, activated by ATP (152). The ADP receptor $P2Y_1$ is a G_q-coupled receptor, leading to activation of phospholipase C. Activation via $P2Y_1$ results in an increase of cytosolic Ca^{2+} levels, shape change and transient aggregation of platelets (20). Another ADP receptor $P2Y_{12}$, a G_i-coupled receptor, is critical for sustained aggregation. Activation of $P2Y_{12}$ potentiates dense granule secretion and the platelet aggregation induced by other pro-thrombotic agonists such as thromboxane A_2 (21). Signaling through G_i, $P2Y_{12}$ inhibits the activity of adenylate cyclase (AC), which suppresses the formation of cyclic AMP (cAMP) and removing an inhibitor of activation (22). However, reduced levels of cAMP are not directly responsible for the downstream effects of $P2Y_{12}$ receptor. G_i signaling also leads to activation of PI3K, and subsequently Akt and Rap1b. Both Akt and Rap1 are important functional effectors downstream of the $P2Y_{12}$ receptor (21). Activation of the $P2X_1$ receptor by ATP leads to fast Ca^{2+} influx (30) and contributes to platelet activation induced by low concentrations of collagen (31). In contrast, platelets also express an A_{2a} receptor, which is activated by adenosine, and activation of A_{2a} inhibits platelet aggregation through AC and results in an increase of cAMP (27). Therefore, it is

likely that the modulation of human platelet aggregation by NT5E in *S. sanguinis* is mediated through the signal transduction of both P2 receptors and A_{2a} receptor.

Using antagonists for P2 receptors and A_{2a} receptor, the contribution of ATP, ADP and adenosine to platelet aggregation was examined by comparing responses to wt and Δ*nt5e* in vitro. Moreover, in response to wt and Δ*nt5e*, the amount of thromboxane A_2, another important secondary agonist secreted from platelets, was also determined.

MATERIALS AND METHODS

Reagents. Adenosine 3'-phosphate 5'-phosphosulfate (A3P5PS), MRS2179, clopidogrel, 2-methylthioadenosine 5′-monophosphate (2MeSAMP), 8-(3-chlorostyryl)caffeine (CSC), SCH58261, indomethacin, and protease inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO). NF449 was purchased from EMD Biosciences (Gibbstown, NJ). Anti-rabbit IgG-horseradish peroxidase (HRP) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and antibodies to Akt, phospho-Akt (Ser^{473} and Thr^{308}) were purchased from Cell Signaling Technology (Danvers, MA). Enhanced chemiluminescence (ECL) reagents were obtained from Amersham Pharmacia Biotechnology (Piscataway, NJ), and all electrophoresis and immunoblotting supplies were from Bio-rad (Hercules, CA).

Bacterial strains and culture conditions. Δ*nt5e* in *S. sanguinis* 133-79 was constructed by allelic exchange as described in chapter 3. *S. sanguinis* 133-79 and the isogenic mutant Δ*nt5e* were routinely grown in Todd Hewitt Broth (TH broth, Difco; Sparks, MD) or on TH agar plates at 37℃ in 5% CO_2. When required, antibiotic was added to the medium at the indicated concentration: erythromycin (Em),10 µg ml^{-1}.

Platelet aggregometry. Strains of *S. sanguinis* were tested for the ability to induce platelet aggregation using fresh platelet-rich plasma (PRP) obtained from a single donor by a method described previously (55). A single donor was used to eliminate variability in platelet response between donors (139). Briefly, each bacterial strain (50 µl suspension containing 4 X 10^9 cells/ml) was then incubated with 450 µl of PRP suspension (4 X 10^8 cells/ml). PRP aggregation was performed at 37℃ with controlled stirring in a record ing aggregometer (model 660, Chronology Corp., Havertown, PA), and the lag time or delay to onset (minutes) was measured. In some experiments, 450 µl of PRP was preincubated with one of the following antagonists prior to stimulation with *S. sanguinis*: A3P5PS, MRS2179, clopidogrel, 2MeSAMP, NF449, CSC, and SCH58261. To estimate inhibition of the lag time to onset of aggregation, the following formula was used: % inhibition = ($t_{observed}$ – t_{blank}) / 12 minutes X 100, where $t_{observed}$ and

t_{blank} are the lag times in minutes with and without antagonists, respectively (53). For all experiments, lag time > 20 min was considered no aggregation (56).

Western immunoblotting. To identify the state of key molecules during the course of platelet aggregation, interactions were stopped by the addition of an equal volume of 2 X stopping solution (20 mM EDTA, 30 mM NaF, 12 mM Na_3VO_4, and 1 X protease inhibitor cocktail). Platelets were pelleted by centrifugation at 8,500 x g for 30 s. Pellets were re-suspended in 500 µl of 1 X RIPA buffer (10 mM Tris•HCl, 158 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 1 mM EDTA, 15 mM NaF, 6 mM Na_3VO_4, and 1 X protease inhibitor cocktail, pH 7.6) and lysed on ice for 10 min. The lysates were centrifuged at 10,000 x g at 4ºC for 15 min. The supernatant was added to an equal volume of 2 X SDS-PAGE loading buffer, boiled for 5 minutes at 95ºC and resolved on 10% (w/v) SDS-PAGE gels. Proteins were transferred onto Trans-Blot nitrocellulose membranes (Bio-Rad, CA) at 15 V for 60 min using a Trans-Blot SD semi-Dry Electrophoretic Transfer Cell (Bio-Rad, CA). Membranes were then probed separately with rabbit anti-Akt and anti-phospho-Akt (1:1,000 dilution), and the results visualized with mouse anti-rabbit HRP IgG (Santa Cruz, CA) and ECL (GE health, NJ). All experiments were performed four times with similar results.

Rap1-GTP assay in platelets. Platelet-bacterial interactions were stopped by the addition of an equal volume of ice-cold acid-citrate-dextrose (ACD), and quickly spin down at 10,000 x g for 30 s. Using an Active Rap1 Pull-down and Detection Kit (Pierce Biotechnology, Rockford, IL), pelleted platelets and bacteria were lysed in the presence of 1 X protease inhibitor cocktail in the 1 X lysis buffer provided and the whole lysates were used to perform affinity precipitation of activated Rap1. The precipitated proteins were analyzed by western blot using antibodies against Rap1. The experiments shown were performed two times with similar results.

Thromboxane B_2 release. Each bacterial strain (50 µl suspension containing 4 X 10^9 cells/ml) was added to 450 µl of PRP (4 X 10^8 cells/ml) and reactions were terminated at the point of full aggregation by addition of an equal volume of ice-cold ACD (pH 4.5) containing 28 µM indomethacin. Platelets were pelleted by centrifugation at 8,500 x g for 30 s and the supernatant from each sample was collected into separate Eppendorf tubes. Levels of released TxB_2 were detected using a TxB_2 assay (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions.

Statistical analysis. Descriptive statistics, including the means and standard errors, were calculated. Statistical analysis of data was performed with Student's *t*-test, or one-way analysis of variance (ANOVA) followed by a Dunnett's post test

using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). The level of significance $\alpha = 0.05$ was considered to be statistically significant.

RESULTS

Inhibition of platelet aggregation by P2Y receptor antagonists in the presence and absence of NT5E. The role of ADP in *S. sanguinis*-induced platelet aggregation was studied using P2Y receptors antagonists: a stable adenosine analogue A3P5PS (153) and a more efficient compound MRS2179 for $P2Y_1$ receptor (154); an AMP analogue 2MeSAMP (155) and an irreversible antagonist clopidogrel for $P2Y_{12}$ receptor (156).

For the $P2Y_1$ receptor, only A3P5PS delayed the onset of platelet aggregation mediated by wt and Δ*nt5e* (Figure 1). At doses greater than 30 μM A3P5PS, platelet aggregation was significantly inhibited in response to both wt ($P < 0.001$, $n = 3$) and Δ*nt5e* ($P < 0.01$, $n = 3$). However, the wt strain was significantly more sensitive to the $P2Y_1$ inhibitor than the *Δnt5e* based on the dose-response curves. With 100 μM A3P5PS, platelet aggregation mediated by wt and Δ*nt5e* were inhibited by $50.5 \pm 4.9\%$ and $21.1 \pm 7.6\%$, respectively ($P < 0.05$, $n = 3$). For each concentration of A3P5PS tested, the magnitude of platelet aggregation was not affected (data not shown).

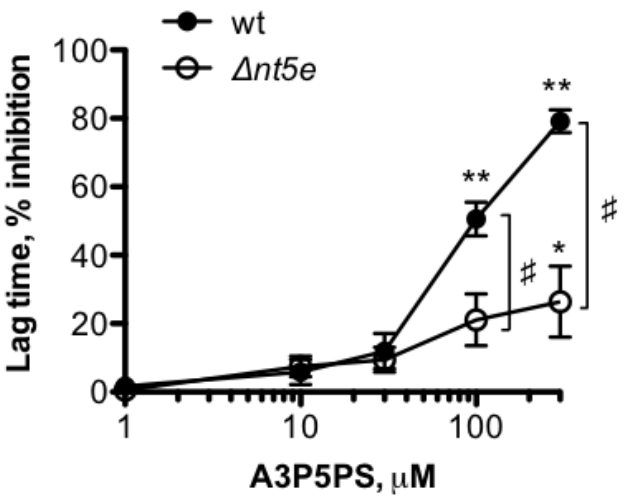


Figure 1. Inhibition of platelet aggregation by $P2Y_1$ receptor antagonist A3P5PS.

1 – 300 μM A3P5PS were added to PRP prior to stimulation with wt and Δ*nt5e*. The results are represented as Mean ± SE, n = 3. Comparison within the group: * P < 0.05, ** P < 0.001; comparison between wt and Δ*nt5e*: # P < 0.05.

When $P2Y_{12}$ receptors were inhibited with either 2MeSAMP or clopidogrel, aggregation lag-time increased in a dose-dependent manner in response to wt or Δ*nt5e* cells (Figure 2A and 2B) without affecting the aggregation magnitude (data not shown). When 100 μM of clopidogrel was used, platelet aggregation was significantly inhibited (Figure 2B; wt: 74.2 ± 3.8%, $P < 0.001$, n = 3; Δ*nt5e*: 42.9 ± 3.4%, $P < 0.001$, n = 3). Interestingly, in the presence of 300 μM clopidogrel or 1 mM 2MeSAMP, the wt strain failed to induce platelet aggregation within 20 min. In contrast, in response to clopidogrel (Figure 2A) or 2MeSAMP (Figure 2B), Δ*nt5e*-mediated platelet aggregation was inhibited by 64.2 ± 4.9% and 70.3 ± 7.7%, respectively. Overall, the two dose-response curves of Δ*nt5e* shifted to the right of wt.

Compared to 100 μM A3P5PS alone, platelet aggregation mediated by wt was significantly prolonged when treated with 100 μM A3P5PS and 100 μM clopidogrel simultaneously (Figure 3; $P < 0.001$, n = 3), and there was no aggregation within 20 min. In response to Δ*nt5e*, platelet aggregation was also significantly inhibited with 100 μM A3P5PS and 100 μM clopidogrel (100 μM A3P5PS: $P < 0.001$, n = 3; 100 μM clopidogrel: $P < 0.05$, n = 3). Therefore, $P2Y_1$ receptor antagonist, A3P5PS, and a $P2Y_{12}$ antagonist, clopidogrel, worked synergistically to inhibit *S. sanguinis*-induced platelet aggregation.

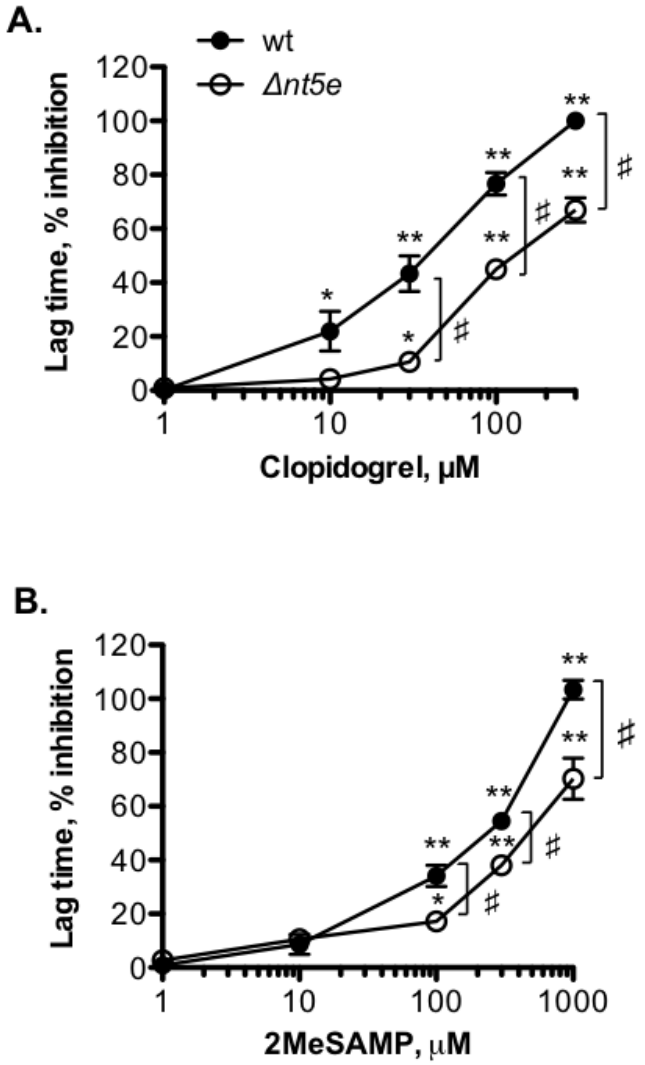


Figure 2. Inhibition of platelet aggregation by $P2Y_{12}$ receptor antagonists clopidogrel and 2MeSAMP.

Antagonists were added to PRP prior to stimulation with wt and Δ*nt5e*. (A) 1 – 300 μM clopidogrel. (B) 1 μM – 1 mM 2MeSAMP. The results are represented as Mean ± SE, n = 3. Comparison within the group: * $P < 0.05$, ** $P < 0.001$; comparison between wt and Δ*nt5e*: # $P < 0.05$.

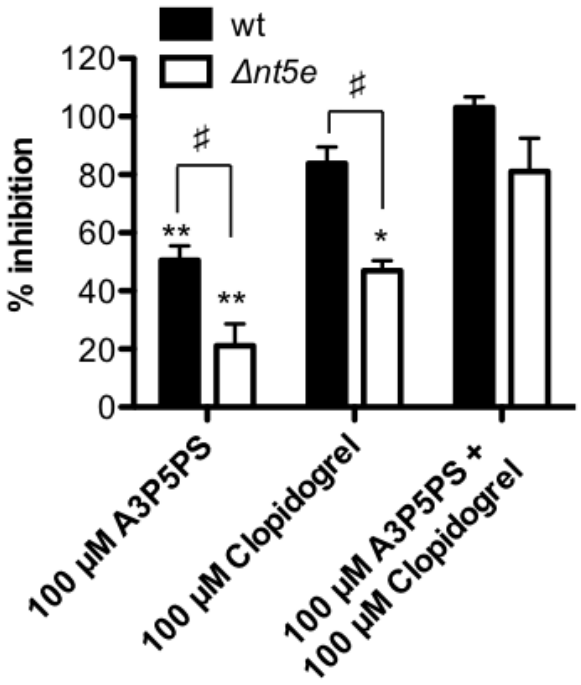


Figure 3. $P2Y_1$ and $P2Y_{12}$ receptor antagonists work synergistically to inhibit *S. sanguinis*-mediated platelet aggregation.

Antagonists were added to PRP prior to stimulation with wt and Δ*nt5e*. (A) 100 μM A3P5PS. (B) 100 μM clopidogrel. (C) 100 μM clopidogrel and 100 μM A3P5PS. The results are represented as Mean ± SE, n = 3. Comparison within the group: * $P < 0.05$, ** $P < 0.001$; comparison between wt and Δ*nt5e*: # $P < 0.05$.

The potential role of ATP was also examined using a potent selective $P2X_1$ antagonist, NF449, an analogue of suramin (157). At all concentrations tested (0.1 – 100 μM), however, there was neither a delay of onset of platelet aggregation nor reduction in magnitude in response to either strain (data not shown).

Acceleration of platelet aggregation by A_{2a} receptor antagonist in the presence of NT5E. Using the A_{2a} receptor xanthine antagonist CSC and a highly selective non-xanthine antagonist SCH58261(158,159), the effect of adenosine on platelet aggregation was examined. Compared with no antagonist control, there was no significant difference in the lag time and magnitude of aggregation with 0.01 - 10 μM CSC (data not shown). However, within the range of 1 to 10 nM SCH58216, aggregation lag time was accelerated only in response to wt (Figure 4). For example, in the presence of 10 nM SCH58216, the aggregation lag time was significantly shorter than control (78.4 ± 2.3 %, $P < 0.001$, $n = 3$).

***S. sanguinis*-induced platelet aggregation involves two waves of Akt phosphorylation.** In response to *S. sanguinis*, phosphorylation of Akt was detected. Upon activation by both wt and Δ*nt5e*, Akt was rapidly phosphorylated at serine and threonine to initiate the response, dephosphorylated before aggregation and rephosphorylated once aggregation begins (Figure 5B).

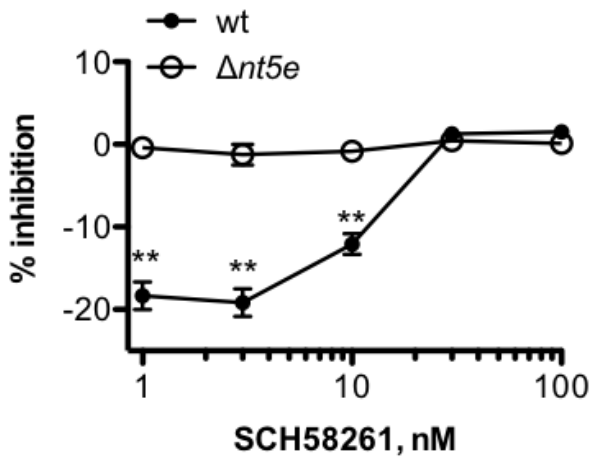


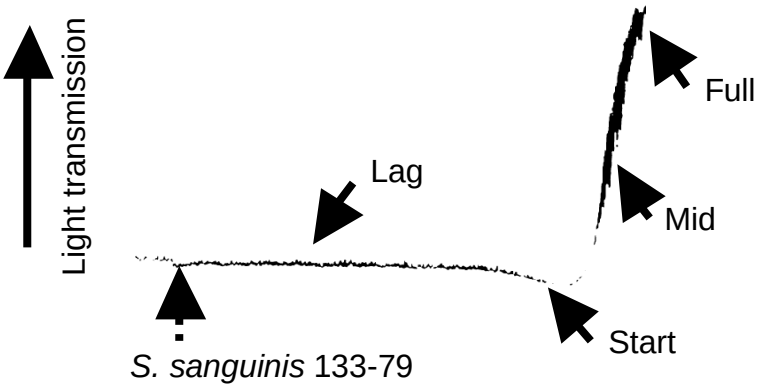
Figure 4. A_{2a} receptor antagonist SCH58261 accelerates platelet aggregation mediated by wt.

1 – 100 nM SCH58261 were added to PRP prior to stimulation with wt and Δ*nt5e*. The results are represented as Mean ± SE, n = 3. Comparison within the group: ** P < 0.001.

NT5E prolongs *S. sanguinis*-induced platelet aggregation by regulating Rap1b activity. In response to *S. sanguinis*, Rap1b was activated. However, in the process of platelet aggregation, the activation of Rap1b differed in the presence and absence of NT5E. Following the addition of wt, Rap1b was de-activated and then re-activated during the lag phase. No activity of Rap1b was detected when platelet aggregation occurred. In contrast, Rap1b was continually active in response to Δ*nt5e* (Figure 6).

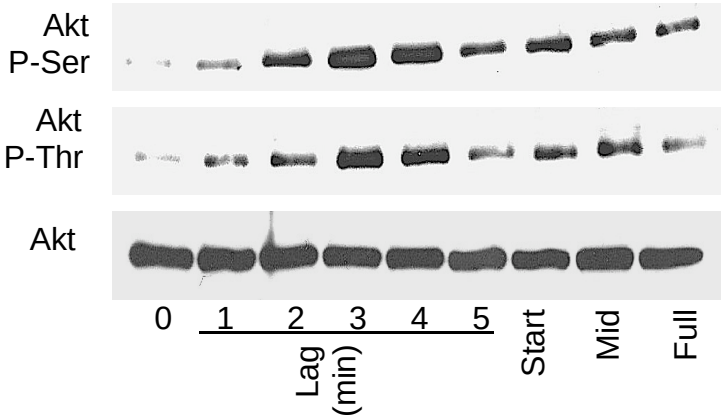
NT5E modulates *S. sanguinis*-induced platelet aggregation independent of TxA_2 release. Upon stimulation with *S. sanguinis*, TxA_2, a positive feedback lipid mediator, is generated from platelets (4,151). Therefore, NT5E might also modulate platelet aggregation through TxA_2 release. In response to wt and Δ*nt5e*, platelets produced comparable amounts of TxA_2 (wt, 20.4 ± 6.7 ng/mL; Δ*nt5e*, 21.6 ± 5.5 ng/mL; n = 4, P = 0.80). Therefore, *S. sanguinis* NT5E modulated platelet aggregation was independent of TxA_2.

A.


Light transmission
Lag
Full
Mid
Start
S. sanguinis 133-79

B.

wt


Akt
P-Ser
Akt
P-Thr
Akt
0 1 2 3 4 5 Start Mid Full
Lag (min)

Δnt5e

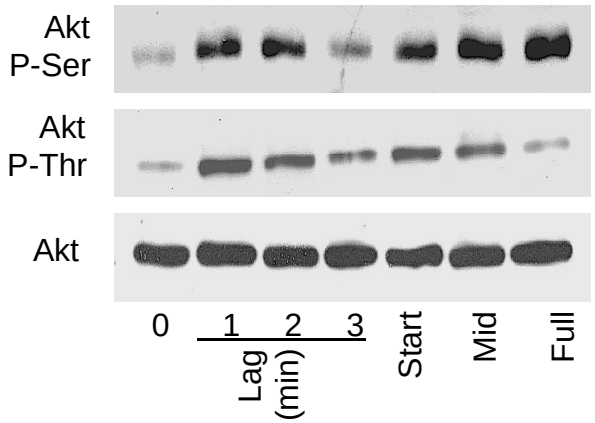

Akt
P-Ser
Akt
P-Thr
Akt
0 1 2 3 Start Mid Full
Lag (min)

Figure 5. *S. sanguinis*-induced platelet aggregation involves two waves of Akt phosphorylation.

(A) Tracing of *S. sanguinis* strain 133-79-induced platelet aggregation from aggregometer. *S. sanguinis* strain 133-79 was added and aggregation measured continuously as light transmission increasing. For western blot analysis, reactions were terminated at 1 min interval during the lag phase prior to aggregation, and at points corresponding to the start of, mid- point of, and full aggregation, as indicated by arrows. (B) Platelets were activated with wt or Δ*nt5e*, and the serine (upper) and threonine (middle) phosphorylation of Akt were shown for the times indicated. The blot probed with anti-Akt antibody was used to confirm equal loading (lower panel). Labels below lower panel refer to aggregation status. Experiment representative of four similar experiments.

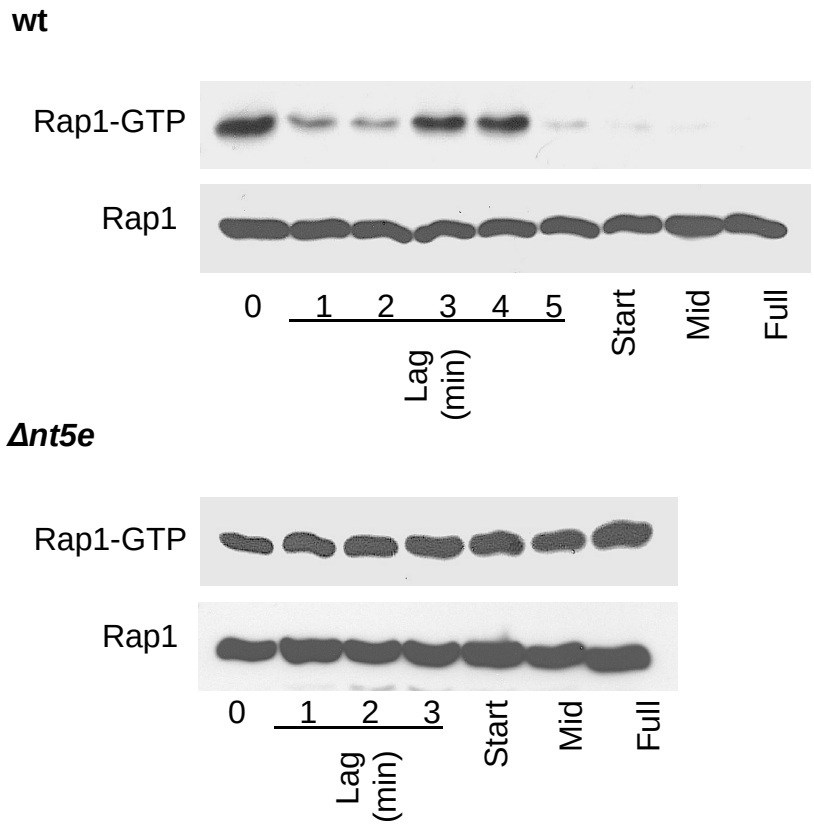


Figure 6. NT5E prolongs *S. sanguinis*-induced platelet aggregation by regulating Rap1b activity.

Platelets were activated with wt or Δ*nt5e*, and the Rap1 activity (Rap1-GTP, upper) was shown for the times indicated. The blot probed with anti-Rap1 antibody was used to confirm equal loading (lower panel). Labels below lower panel refer to aggregation status. Experiment representative of two similar experiments.

DISCUSSION

Previously, we identified and characterized NT5E, a cell-surface enzyme on *S. sanguinis*, which can metabolize extracellular ATP, ADP and AMP (chapter 3). When platelets release their dense granules in response to *S. sanguinis*, the granule contents including equimolar amounts of ATP and ADP are secreted into the surrounding environment. Reflecting the contribution of hydrolyzed nucleotides to modulation of platelet aggregation, a Δ*nt5e* strain induced human platelet aggregation more rapidly than its isogenic wt strain. Here, we studied how *S. sanguinis* NT5E modulates platelet aggregation.

Both ADP removal and adenosine generation slows down the onset of platelet aggregation. Thus, *S. sanguinis* NT5E potentially modulates platelet aggregation through ADP and adenosine. Using specific pharmacological antagonists for their corresponding receptors, the role of ADP and adenosine in *S. sanguinis*-induced platelet aggregation were distinguished.

To obtain more physiological responses, platelet aggregation was performed with platelet rich plasma (PRP) instead of washed platelets. Other workers used washed platelets to monitor the phosphorylation of key signaling intermediates (160). Plasma contains abundant human serum albumin and virtually all other proteins in the blood, providing physiologically representative protein binding sites and opportunities for intermolecular interactions. Hence, the efficiency of

the antagonists in PRP can be differently affected when compared to washed platelets (161). Thus, receptor-specific antagonists from different families were tested.

The aggregation response to bacteria like *S. sanguinis* is different from other agonists. It is an all-or-nothing response. Also, unlike other agonists, there is a distinct lag time before aggregation occurs (91). When induced by the strains we have studied, platelet aggregation in vitro occurs after a lag time of 1.5 to 15 min depending on the platelet donor and the strain and dose of *S. sanguinis* (55,56,139). When treated with apyrase, an ADP hydrolase, PRP showed only an increase in lag time in response to wild-type *S. sanguinis*, reflecting the hydrolysis of the secreted platelet agonist, ADP (91). The extent of platelet aggregation was unaffected. Blocking of ADP receptors was, therefore, expected to prolong the lag times to platelet aggregation.

In a dose-dependent manner, the $P2Y_1$ antagonist, A3P5PS (Figure1), and $P2Y_{12}$ receptor antagonists, clopidogrel and 2MeSAMP (Figure 2), prolonged the lag time to platelet aggregation without affecting aggregation magnitude. As expected, the effective concentration for those antagonists were 1000-fold higher in plasma than reported for washed platelets (155,162,163), reflecting non-specific interactions with plasma proteins. For example, PRP pretreated with clopidogrel ($\leq$ 100 μM) showed no antiaggregatory effects when stimulated with 6

μM ADP(163). Consistent with previous studies (90), however, PRP preincubated with clopidogrel followed by *S. sanguinis* showed an inhibitory effect. In response to *S. sanguinis*, PRP aggregation appeared to depend, therefore on the lower concentrations of ADP released from activated platelets.

In PRP, the wt and Δ*nt5e* strains showed different dose-response inhibition curves in the presence of antagonists. As shown in figures 1 and 2, the inhibition curves for Δ*nt5e* shifted to the right of wt, reflecting an ADP concentration-dependent response. Hence, the platelets acted as if the amount of plasma ADP was higher in the absence of NT5E. In addition, a synergistic inhibitory effect on platelet aggregation was seen when 100 μM of A3P5PS and 100 μM of clopidogrel were used to block both $P2Y_1$ and $P2Y_{12}$ concurrently (Figure 3) (159,164). Hence, ADP signaling through $P2Y_1$ and $P2Y_{12}$ appeared to play an important role in determining the lag times.

Activation of another P2 receptor, $P2X_1$, by ATP results in rapid calcium influx and contributes to platelet activation in response to low concentrations of collagen (30,31). In response to *S. sanguinis*, platelet aggregation is triggered by the cell wall-anchored platelet-aggregation-associated protein (PAAP), which is a rhamnose-rich glycoprotein containing a collagen-like platelet-interactive domain (6,165). By analogy to the platelet response to collagen, *S. sanguinis*-induced platelet aggregation might also involve ATP signaling.

NF449, a potent $P2X_1$ receptor antagonist, did not have significant affect on platelet aggregation, suggesting that the $P2X_1$ receptor was not a contributor to platelet aggregation triggered by *S. sanguinis*. Alternatively, NF449 might be complexed with serum albumin or other proteins in the plasma minimizing the effective dose. On the other hand, ATP and its analogues are also competitive antagonists at both $P2Y_1$ (28) and $P2Y_{12}$ (29) receptors. Therefore, ATP might inhibit *S. sanguinis*-induced aggregation through ADP receptors.

In contrast, SCH58216, an A_{2a} receptor antagonist, accelerated platelet aggregation triggered by wt but not Δ*nt5e*. Since deletion of NT5E completely abolished the AMPase activity on *S. sanguinis* 133-79, Δ*nt5e* cannot hydrolyze AMP to adenosine (chapter 3). In the presence of the A_{2a} receptor antagonist, therefore, NT5E appeared to produce a higher concentration of adenosine in PRP.

The $P2Y_{12}$ receptor is crucial to several platelet functions based upon experimental use of $P2Y_{12}$ receptor antagonists and $P2Y_{12}$ deficient mice (166). Activation of $P2Y_{12}$ receptor results in dense granule secretion and fibrinogen-receptor activation, which is a central event in platelet aggregation (167,168). Signaling through $P2Y_{12}$ receptor leads to reduction of cAMP, activation of potassium channels, PI3K, and subsequently Akt, and Rap1b (169).

Upon activation by *S. sanguinis*, there were two waves of Akt phosphorylation, and activation of Rap1b, both of which depend on the ADP receptor $P2Y_{12}$. However, when NT5E is present, the ADP concentration is reduced in proximity to the platelet, leading to intermittent Rap1b activation. Therefore, NT5E indirectly modulates platelet aggregation via signaling through Rap1b.

Two secondary messengers, cAMP and cytosolic Ca^{2+}, are also important early intracellular mediators of platelet activation (170,171). Platelets require the presence of extracellular Ca^{2+} to be induced to aggregate by *S. sanguinis* (55). Furthermore, preincubation of platelets with 0.01 to 1 µM PGE_1, which increases intracellular cAMP (172), completely inhibited *S. sanguinis*-induced platelet aggregation (data not shown). Given these data, therefore, changes in platelet cytosolic Ca^{2+} and cAMP were not monitored over time.

The $P2Y_{12}$ receptor also plays a crucial role in ADP-mediated generation of TxA_2 (173). Platelet aggregation in response to *S. sanguinis* was inhibited by indomethacin, a nonselective inhibitor of cyclooxygenase 1 and 2, suggesting that a product, TxA_2 contributes to the aggregation process (151). Although the $P2Y_{12}$ receptor contributes to the platelet response to *S. sanguinis*, we showed that the amount of TxA_2 generated from platelets in response to wt and Δ*nt5e* is

comparable. Hence, NT5E modulates *S. sanguinis*-induced platelet aggregation independently of TxA_2, which signals and activates through other receptors.

In conclusion, *S. sanguinis* NT5E slows down the onset of platelet aggregation through both ADP removal and adenosine production. This study further elucidates crucial roles of ADP and adenosine in *S. sanguinis*-induced platelet aggregation, which might provide insights into novel therapeutics for cardiovascular infectious diseases, such as IE.

CHAPTER 5

ECTO-5'-NUCLEOTIDASE CONTRIBUTES TO THE VIRULENCE OF *S. SANGUINIS* IN RABBIT INFECTIVE ENDOCARDITIS

The ecto-5'-nucleotidase (NT5E) deletion mutant (Δ*nt5e*) of *Streptococcus sanguinis* 133-79 induces platelets to aggregate faster than its isogenic wild-type strain (wt). NT5E, a cell-surface anchored enzyme, can hydrolyze adenosine triphosphate (ATP) to adenosine diphosphate (ADP), adenosine monophosphate (AMP), and subsequently adenosine. ATP is a proinflammatory molecule, whereas adenosine is immunosuppressive. Thus, NT5E is potentially involved in bacterial-host interactions and may produce adenosine from nucleotides released from platelet dense granules to reduce the clearance of infecting bacteria. Using a rabbit endocarditis model, the virulence of wt, Δ*nt5e* and *nt5e*+ were compared in vivo. Upon intravenous infection of rabbits with prior heart valve injury, the mass of the platelet vegetations correlated with the recovered bacterial CFUs when all three strains were included in the analysis ($R^2 = 0.72$). When rabbits were challenged with Δ*nt5e*, the mass of vegetations and the recovered bacterial CFUs were lower than after infection with wt and *nt5e*+, which were similar to one another. NT5E appears, therefore, to contribute to the virulence and survival of *S. sanguinis* in vivo by removing ATP and generating adenosine, which would dampen the inflammatory response to infecting bacteria.

INTRODUCTION

Streptococcus sanguinis, an oral commensal bacterium, is the leading cause of infective endocarditis (IE) (1,8). In an animal model, the abilities of *S. sanguinis* to adhere to and aggregate platelets correlate with the severity of IE (87).

We identified ecto-5'-nucleotidase (NT5E), a cell wall–anchored enzyme on *S. sanguinis* that hydrolyzes adenosine tri- (ATP), di- (ADP), and mono-phosphate (AMP) to adenosine, as a modulator of platelet aggregation in vitro (chapter 3). In response to *S. sanguinis* and other agonists, ATP and ADP are released from platelets in equimolar concentrations. *S. sanguinis* NT5E slows down the onset to platelet aggregation by hydrolyzing ADP, a platelet agonist, and generating adenosine, a platelet antagonist (chapter 4). Among *S. sanguinis* strains, the whole cell NT5E activities vary, resulting in different amounts of hydrolyzed adenine nucleotide products (chapter 3). Strains that promoted aggregation after the shortest lag time to onset showed the greatest specific activity of NT5E. Hence, NT5E may contribute to the bacterial aggregation phenotype.

NT5E might also contribute to the virulence of *S. sanguinis* in vivo. During bacterial infections, ATP and adenosine are both important immune regulators. Extracellular ATP can trigger the release of proinflammatory cytokines such as IL-1β and IL-12 (105,174). ATP-stimulated cytokine release from mammalian cells can be inhibited by CD39, an ecto-ATPDase, which hydrolyzes ATP and

ADP to AMP; the expression of CD39 on T_{reg} cells has been linked to decreased activation of dendritic cells (175,176). CD73, a mammalian ecto-5'-nucleotidase, can hydrolyze AMP to produce adenosine, which inhibits effector T-cell activation and suppresses proinflammatory cytokine expression (177). Adenosine is a potent immunosuppressive molecule, which completely suppresses immune responses (106). Hence, mammalian ecto-nucleotidases can attenuate the inflammatory response by hydrolyzing pro-inflammatory ATP and producing immunosuppressive adenosine.

Immunosuppressive adenosine can also by generated by AMPases on pathogens like *Staphylococcus aureus* and *Bacillus anthracis* and is required for escape from phagocytic clearance in the blood (107). Successful host defense is a balance of pro- and anti-inflammatory mediators. Therefore, the consumption of ATP as well as generation of adenosine by *S. sanguinis* NT5E could potentially mimic the action of mammalian ecto-ATPDase and ecto-5'-nucleotidase and influence the immune response during infection.

To further study the role of NT5E in vivo, wt, Δ*nt5e*, and a *nt5e* complementary strain (*nt5e*+) were inoculated separately by intravenous infusion into different groups of rabbits prepared for experimental endocarditis of the aortic valve (178). To demonstrate the virulence of NT5E in experimental endocarditis, the mass of vegetations and bacteria counts in the vegetations were both determined.

MATERIALS AND METHODS

Bacterial strains and culture conditions. In *S. sanguinis* 133-79, Δ*nt5e* was constructed by allelic exchange and Δ*nt5e* was also complemented to obtain *nt5e*+ as described in chapter 3. *S. sanguinis* 133-79 and its isogenic mutants Δ*nt5e* and *nt5e*+ were routinely grown in Todd Hewitt Broth (TH broth, Difco; Sparks, MD) or on TH agar plates at 37℃ in 5% CO_2. When required, antibiotics were added to the medium at the indicated concentrations: erythromycin (Em), 10 μg ml^{-1}; kanamycin (Km), 400 μg ml^{-1}.

Experimental endocarditis model. Injury-induced experimental endocarditis was initiated by placement of a catheter into the left side of the heart in healthy male adult New Zealand White rabbits, weighing 2 to 3 kg (obtained from Bakkom Rabbitry, Red Wing, MN) essentially as described previously (87,178). The catheter was retained in place for 2 hours to damage the heart valve and then removed. After closure of the neck incision, viable *S. sanguinis* was injected intravenously via the marginal ear vein. A total of 24 rabbits were inoculated with 1 X 10^9 of *S. sanguinis* wt (n = 9 rabbits), Δ*nt5e* (n = 10) and *nt5e*+ (n = 5). After four days, the animals were euthanized, hearts were removed, aortic valves were excised and vegetations were weighed. To determine infecting bacterial colony forming units (CFUs), vegetations were homogenized and plated onto TH agar with and without the appropriate antibiotics to learn whether the resistance markers were lost during the infection

period. For Δ*nt5e* group, the CFUs on TH plates with or without antibiotics were comparable (P = 0.92). In contrast, less than 1% of the bacteria enumerated from the *nt5e*+ group retained the complemented plasmid. When vegetations were not visualized, all of the aortic valve leaflets were scraped and cultured to enumerate the bacteria colonizing the valves. Since all bacteria recovered from the aortic valve potentially contributed to the infection and vegetation formation, CFUs on TH plates were used for statistical analysis. All experiments were conducted under the established guidelines and with approval of the University of Minnesota Institutional Animal Care and Use Committee.

Statistical analysis. Descriptive statistics, including the means and standard deviations, were calculated. Total CFUs were converted to $\log_{10}$ values prior to statistical analysis using Welsh-corrected *t* tests with GraphPad Prism 5 (GraphPad Software, La Jolla, CA). The level of significance $\alpha = 0.05$ was considered to be statistically significant.

RESULTS

NT5E contributes to the virulence of *S. sanguinis* in rabbit infective endocarditis. To investigate the contribution of NT5E to the virulence of *S. sanguinis* in infective endocarditis, rabbits were infected by intravenous inoculation with wt, Δ*nt5e*, or *nt5e*+. The resulting vegetations ranged from non-apparent (Figure 1A) to macroscopic lesions (Figure 1B and 1C).

Bacterial infections of the heart valves were confirmed by culturing homogenized vegetations and enumerated as CFUs on agar plates. Four days after inoculation with 1 x 10^9 CFU of *S. sanguinis*, bacterial cells in the vegetations ranged from 10^3 to 10^9 per ml of homogenate, and the vegetative masses correlated with the recovered bacterial CFUs (Fig. 2A; $R^2 = 0.72$, n = 22). Five of 22 infected rabbits had no visible vegetations, which might due to insignificant injury caused during catheter placement. Hence, total recovered bacterial CFUs were used to indicate the relative ability of each strain to colonize and proliferate on the heart valve.

As expected, 8 out of 9 rabbits infected with wt formed aortic vegetations with a mean mass of 18.7 mg and mean recovered bacterial load of 0.9 X 10^9 CFU. Similarly, after infection with *nt5e*+, vegetations formed in 4 of 5 rabbits with a mean mass of 13.4 mg and mean bacterial load of 1.0 X 10^9 CFU on TH plates, and 0.7 X 10^6 CFU on TH plates with appropriate antibiotics. In comparison, three of 10 rabbits injected with Δ*nt5e* showed no vegetations. Two rabbits died before euthanasia and were excluded from statistical analysis. The mean weight of vegetations and bacterial load in rabbits challenged with Δ*nt5e* was 5.5 mg and 0.3 X 10^7 CFU on both selective and non-selective plates, respectively, which were the lowest among these three groups. Taken together, Δ*nt5e*

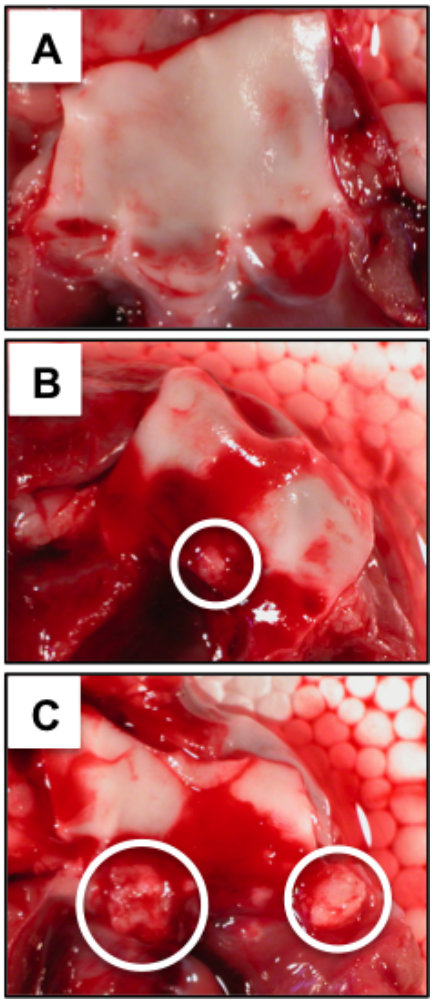


Figure 1. Vegetations on the aortic valves of rabbits.

(A) Aortic valve of a rabbit infected with Δ*nt5e*. The aortic valve is composed of three leaflets and no visible vegetations were found. The bacterial CFU was 2.5 X 10^3. (B) Aortic valve of a rabbit infected with *nt5e*+, with one vegetation in the center leaflet (white circle). The bacterial CFU was 3.3 X 10^8. (C) Two vegetations were found on center and right leaflets (white circles), respectively. The bacterial CFU was 3.4 X 10^9.

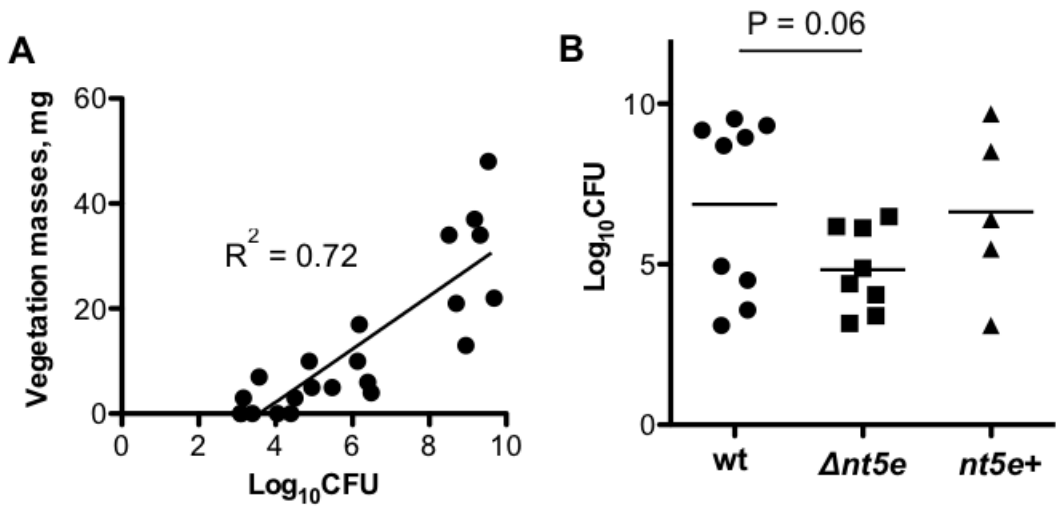


Figure 2. Vegetation weight and bacterial load in a *S. sanguinis* rabbit endocarditis model.

(A) Plot of vegetation bacterial load (total CFU) versus vegetation mass in a rabbit endocarditis model. After the catheter was removed, viable *S. sanguinis* cells (1 X 10^9 CFU) were injected intravenously after catheter removed. All vegetations on the aortic valve of each rabbit were pooled to obtain the vegetation weight and bacterial load (on TH plate). When no vegetations were found, the valves were scraped with a blade and plated to determine the valve bacterial load. $R^2 = 0.72$ (n = 22) indicated that there is a correlation between the bacterial load and vegetation masses. (B) Bacterial loads in the rabbit endocarditis model, enumerated as $\log_{10}$ total CFU 4 days after infection. Horizontal bars represent mean CFUs in each cohort.

infected rabbits developed smaller aortic vegetations with lower bacterial CFUs than rabbits challenged with the wt (vegetation: P = 0.01; CFU: P = 0.06) and *nt5e*+ (vegetation: P = 0.06; CFU: P = 0.11). The Δ*nt5e* group showed an approximately 100-fold reduction in mean bacterial CFUs as compared with the wt group (Figure 2B).

DISCUSSION

Our in vitro studies suggested that NT5E could play a role in streptococcal IE. Using a rabbit endocarditis model, we compared the virulence of wt, Δ*nt5e*, and *nt5e*+ strains in vivo. Virulence was reflected by the weight of cardiac vegetations and bacterial burden (87,178). The mass of the vegetations was directly related to the CFUs of bacteria colonizing the vegetations (R^2 = 0.72, n = 22) when total CFU of all strains were plated on non-antibiotic-containing plates and included in the statistical analysis. In Δ*nt5e* challenged rabbits, both vegetation masses and bacterial CFUs were lower than wt and *nt5e*+ infected rabbits. The CFU counts of *Δnt5e* group were 100-fold lower than the others, and the differences approached statistical significance. Based on these data, *nt5e* appears to be responsible for the increased virulence of the wild-type and complemented strains.

Differences in vegetation formation and recovery of CFUs from the heart valves of infected rabbits may stem from the reduced efficiency of bacterial clearance from heart valves when NT5E was expressed, causing more bacteria to colonize. The efficacy of clearance may reflect the affects of adenosine on phagocytosis of bacteria attempting to colonize the heart valves. Adenosine is a general immunosuppressive and anti-phagocytic mediator. In the presence of *nt5e* (i.e., wt and *nt5e*+ strains), colonization would be facilitated because the concentrations of locally available adenosine would be higher than in vegetations from the Δ*nt5e* group. *S. sanguinis* 133-79 NT5E can break down ATP to ADP, AMP and finally inorganic phosphate and adenosine (chapter 3). Interestingly, ATP is a proinflammatory molecule, while adenosine is a potent immunosuppressive molecule. Therefore, NT5E potentially promotes bacterial survival through ATP removal and adenosine generation.

nt5e could also play a direct role in the formation of vegetations in vivo. The Δ*nt5e* strain induced platelet aggregation faster than *S. sanguinis* 133-79 isogenic wild-type strain, without affecting bacterial-platelet adhesion. Perhaps working in synergy with the anti-inflammatory actions of adenosine, more rapid platelet aggregation by the Δ*nt5e* strain may place platelet microbicidal proteins in proximity to infecting bacteria sooner and more effectively than when *nt5e* is expressed. By reducing the local infecting inoculum, the mass of the vegetation

and eventual bacterial load will decrease. Therefore, *nt5e* may contribute to the bacterial survival and persistence in the vegetation.

In some rabbits, lung congestion was observed. Formation of very large aggregates of bacteria and platelets would be expected to obstruct small capillaries, such as those in the lungs (87,179). However, an association of lung congestion with *nt5e* or size of vegetation was not found (data not shown).

One technical consideration deserves discussion. Note that on antibiotic-containing and non-antibiotic plates, the bacterial counts from rabbits infected with Δ*nt5e* were similar. Since rabbits infected with *nt5e*+ showed significantly different post-infection bacterial counts, the antibiotic resistance was cured or lost without selective pressure from appropriate antibiotics. However, the vegetation masses and bacterial CFUs recovered from non-antibiotic plates of the *nt5e*+ group were comparable to the wt group. The *nt5e*+ was suggested to gradually lose the complemented plasmid during the course of infection. These results further supported the importance of NT5E for the persistence of *S. sanguinis* in the initiation of heart valve infection.

Collectively, NT5E was shown to contribute to the virulence of *S. sanguinis* in rabbit IE. Widely distributed on bacterial cells, NT5E might also be a critical virulence factor for the survival of bacterial pathogens in the bloodstream.

CHAPTER 6

FINAL DISCUSSION

Streptococcus sanguinis, an oral commensal bacterium, is the leading cause of infective endocarditis (IE) (1,8). In a rabbit model, the abilities of *S. sanguinis* to adhere to and activate platelets are correlated with the increased severity of IE (1,8). In response to *S. sanguinis*, platelet activation is associated with secretion of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) from dense granules (90). The extracellular ADP is a potent platelet agonist and amplifies platelet aggregation induced by other pro-thrombotic agonists (140), whereas, the final product of hydrolysis of adenine nucleotides, adenosine, is a platelet aggregation antagonist (27). Here, we show that cell surface ecto-5'-nucleotidase (NT5E) of *S. sanguinis* can convert adenine nucleotides ATP to ADP, adenosine monophosphate (AMP) and finally adenosine. Therefore, we hypothesized that *S. sanguinis* ecto-5'-nucleotidase modulates platelet aggregation.

A *nt5e* deletion mutant of *S. sanguinis* 133-79 (Δ*nt5e*) showed significantly shorter lag time to the onset of platelet aggregation than the wild-type (wt) and *nt5e* complemented (*nt5e*+) strains. However, Δ*nt5e* adhered to human platelets indistinguishably from the wt and *nt5e*+ strains. Hence, NT5E modulates *S. sanguinis*-induced platelet aggregation independent of platelet-bacterial adhesion in vitro.

By hydrolyzing extracellular adenine nucleotides released from activated platelets, NT5E potentially modulates platelet aggregation through ADP and adenosine. To further elucidate the mechanism, using specific pharmacological antagonists, we distinguished the roles of ADP and adenosine on streptococcal-platelet interactions. In a dose-dependent manner, antagonists for ADP receptors $P2Y_1$ and $P2Y_{12}$ prolonged lag times to platelet aggregation mediated by both wt and Δ*nt5e* strains. However, the wt strain is more sensitive to the antagonists than Δ*nt5e*, reflecting an ADP-dependent response. In contrast, an antagonist of adenosine A_{2a} receptor only accelerated platelet aggregation mediated by the wt strain where NT5E was present. Downstream of the $P2Y_{12}$ receptor, *S. sanguinis* induced-platelet activation involves two waves of Akt phosphorylation and activation of Rap1b. However, in response to wt, Rap1b was de-activated and then reactivated during the lag phase prior to aggregation, whereas, Rap1b was continually active in response to Δ*nt5e*. Through these pathways, nevertheless, *S. sanguinis* NT5E slows down the onset of platelet aggregation by removing ADP and generating adenosine.

In addition, strains of *S. sanguinis* have shown different platelet-interactivity phenotypes (56). Hence, we also examined *S. sanguinis* whole cell surface enzymatic activities for hydrolysis of adenine nucleotides among strains with different phenotypes. Interestingly, the activities on strains with different aggregation phenotypes varied. Generally, the enzymatic activities on

Adh+Agg+ strains were the highest among three different platelet interactivity phenotypes, suggesting that NT5E contributes to the bacterial-platelet interactivity phenotype in concert with other factors such as adhesin expression and PAAP. To better define the role of NT5E, more *S. sanguinis* strains need to be characterized and multiple variables must be included into the regression analysis.

Using a rabbit endocarditis model, the virulence of wt, Δ*nt5e* and *nt5e*+ were compared in vivo. Upon intravenous infection of rabbits with prior heart valve injury, the mass of the platelet vegetations correlated with the recovered bacterial loads. When rabbits were challenged with Δ*nt5e*, the mass of vegetations and the recovered bacterial loads were lower than after infection with wt and *nt5e*+, which were similar to one another. NT5E appears, therefore, to contribute to the virulence and survival of *S. sanguinis* in vivo by removing ATP, a pro-inflammatory molecule, and generating adenosine, an immunosuppressive molecule, both of which would dampen the inflammatory response to infecting bacteria. Perhaps working in synergy with the anti-inflammatory actions of adenosine, more rapid platelet aggregation by the Δ*nt5e* strain may place platelet microbicidal proteins in proximity to infecting bacteria sooner and more effectively than when *nt5e* is expressed. By reducing the local infecting inoculum, the mass of the vegetation and eventual bacterial load will decrease.

In conclusion, streptococcal NT5E modulates *S. sanguinis*-induced platelet aggregation and contributes to the virulence of streptococci in IE.

Proposed mechanism of NT5E in *S. sanguinis*-platelet interaction

Based on our findings, we propose mechanisms for NT5E in *S. sanguinis*-platelet interaction (Figure 1). In response to *S. sanguinis*, platelets are activated, and secrete TxA_2 as well as release of equimolar of ADP and ATP from dense granules (90). ADP and TxA_2 are both platelet agonists, which further propagate the aggregation cascade (140). On the surface of *S. sanguinis*, NT5E obtains proximity to hydrolyze ATP and ADP released from platelets. Through removing ADP, a platelet agonist, and generating adenosine, a platelet antagonist, NT5E slows down *S. sanguinis*-induced platelet aggregation in vitro.

This mechanism may also be applicable to streptococcal-immune cell interactions (Figure 2). ATP and adenosine receptors are widely distributed on immune cells, including T cell, B cell, and neutrophil (112). Extracellular ATP is a proinflammatory molecule (104,105,175,176), whereas adenosine is an immunosuppressive molecule (106,177). On infected heart valves, *S. sanguinis* NT5E removes ATP and generates adenosine, which could also help *S. sanguinis* to survive in the blood. On the other hand, NT5E slows down the *S.*

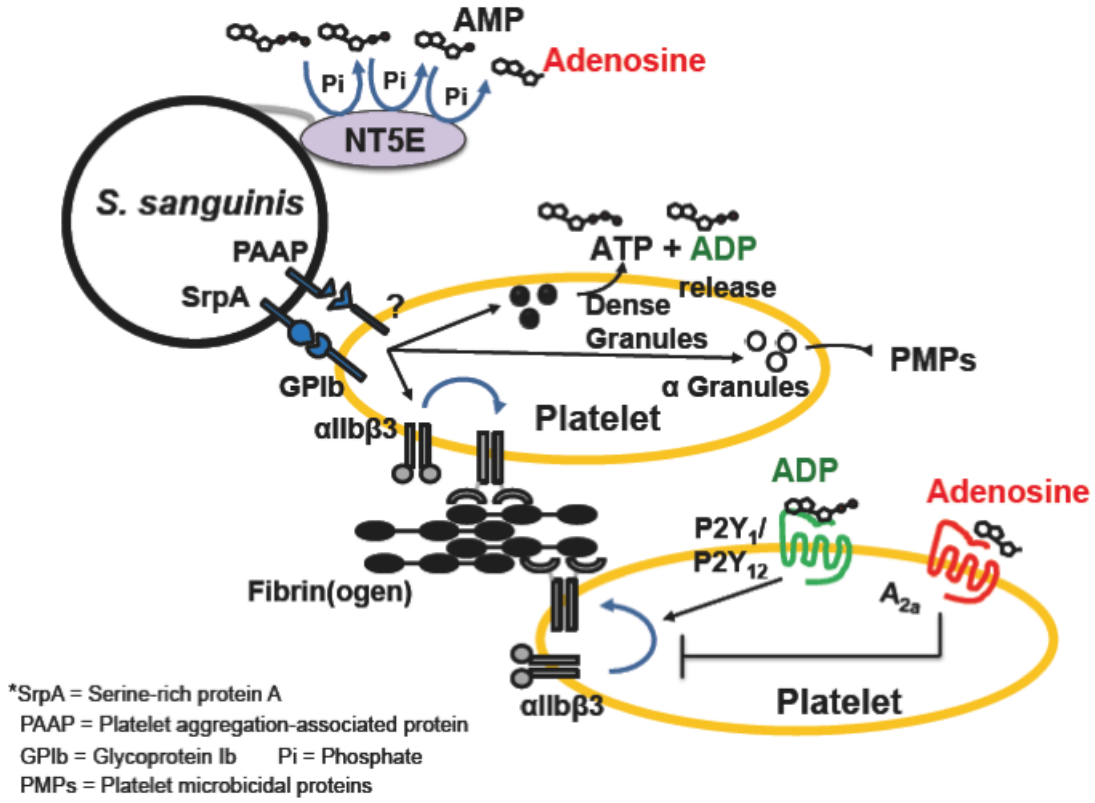


Figure 1. Schematic features for proposed mechanisms of NT5E in *S. sanguinis*-platelet interaction.

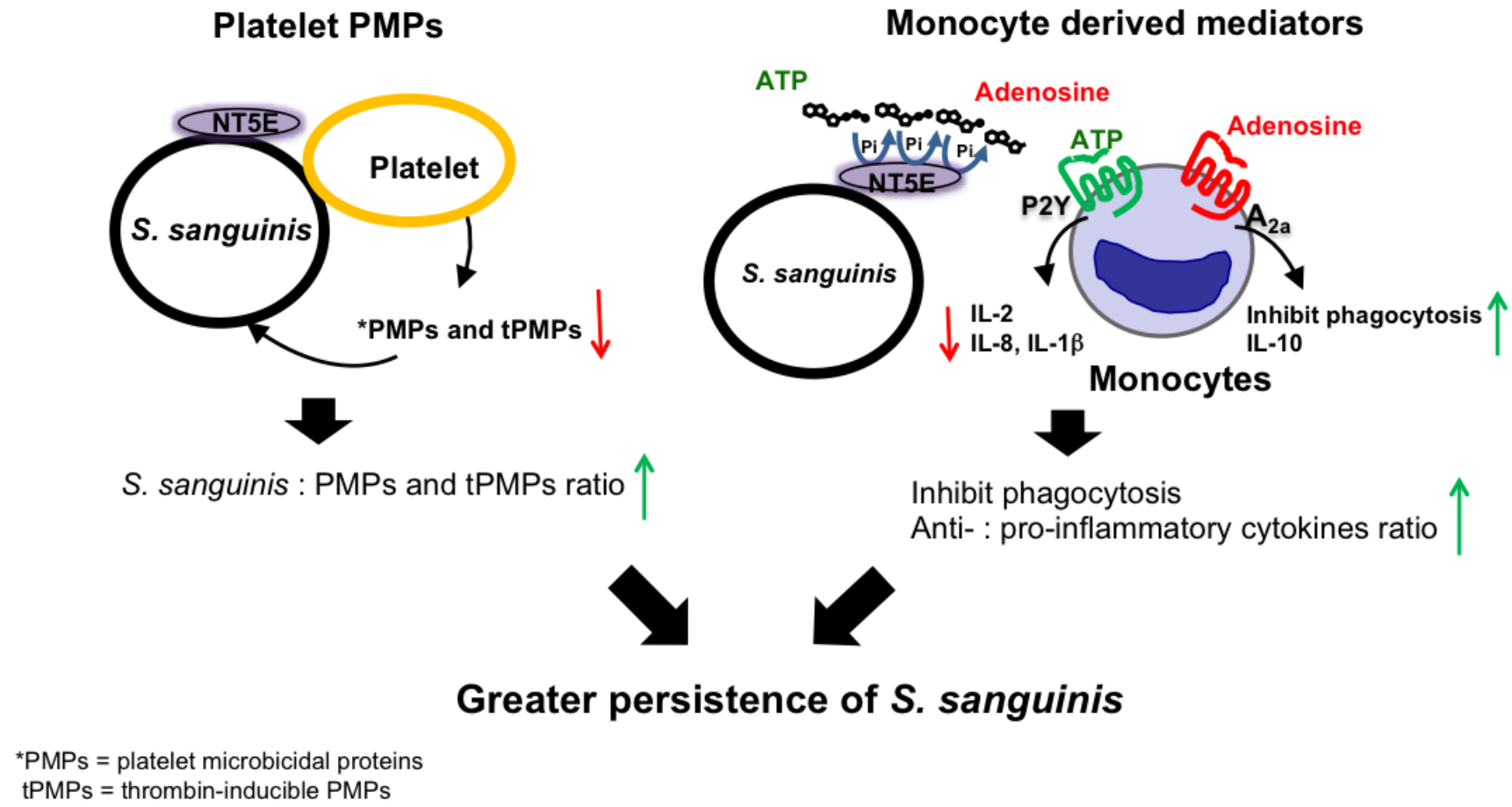


Figure 2. Reconciling in vitro results with *S. sanguinis* interactions in experimental IE in vivo.

sanguinis-induced platelet aggregation, which may reduce the platelet microbicidal proteins (PMPs) in proximity to the bacteria, resulting in higher infecting bacterial density. During the course of infection, therefore, NT5E may promote the persistence of *S. sanguinis* through reducing PMPs and suppressing immune responses, which result in bigger vegetation formation on the heart valve.

Conclusions

By hydrolyzing AMP to adenosine, mammalian NT5E (CD73) is involved in very diverse biological processes including neurotransmission, platelet aggregation, and modulation of the immune response (106,177). Here, we showed for the first time that bacterial NT5E modulates bacterial-platelet interaction in vitro. Using an animal model, we also showed that NT5E contributes to the virulence of streptococci in IE. Taken together, these findings expand our knowledge on bacterial-host interactions, which may help in understanding bacterial-host interaction and potentially provide information for the development of novel therapeutics.

Future directions

We showed that *S. sanguinis* NT5E modulates platelet aggregation through ADP removal and adenosine generation in vitro. For adenine nucleotide hydrolysis, cell surface enzymatic activities on *S. sanguinis* strains differ, which may contribute to determine the platelet interactivity phenotype. Since NT5E is widely distributed (94), these findings may be a common mechanism and applicable to other bacterial pathogens, such as staphylococci and enterococci, which invade the bloodstream (Table 1). This study focused on the cell surface enzymatic activities of NT5E. We need to show the activities of recombinant NT5E in platelet aggregation. This information will help to further elucidate the mechanism whereby NT5E modulates platelet aggregation. To extend the scope of studies, we also showed that *nt5e* contributes to the virulence of *S. sanguinis* in a rabbit endocarditis model. We need to learn whether the increased pathogenicity of *S. sanguinis* is attributed to slower PMPs production from platelets or ATP breakdown and adenosine synthesis by NT5E in the blood. Nevertheless, identifying the role of NT5E in facilitating streptococcal survival in blood will broaden our understanding of the mechanisms of bacterial-host interaction. More importantly, NT5E might be a potential drug target for treating and preventing serious cardiovascular infections.

Table 1. Gram-positive bacteria harboring *nt5e* gene

Organism	Vascular diseases associated	Accession No.[a]	Substrates	References
Bacillus subtilis	Hemorrhage	multiple	AMP, ADP, ATP	(180)
B. anthracis	Hemorrhage	BAS4031	AMP	
Staphylococcus aureus	IE	A6QD62	AMP	(107)
Streptococcus sanguinis	IE	A3CN82	AMP, ADP, ATP	This study
S. gordonii	IE	A8AXM1	ND	
Enterococcus faecalis	IE	Q839U0	ND	

[a]Available from GenBank.

[b]ND, not determined.

BIBLIOGRAPHY

1. Moreillon P, Que Y-A. Infective endocarditis. Lancet. 2004 Jan 10;363(9403):139-149.

2. Franchini M, Veneri D. Helicobacter pylori infection and immune thrombocytopenic purpura: an update. Helicobacter. 2004 Aug;9(4):342-346.

3. Levi M, Keller TT, van Gorp E, ten Cate H. Infection and inflammation and the coagulation system. Cardiovasc. Res. 2003 Oct 15;60(1):26-39.

4. Kerrigan SW, Douglas I, Wray A, Heath J, Byrne MF, Fitzgerald D, et al. A role for glycoprotein Ib in Streptococcus sanguis-induced platelet aggregation. Blood. 2002 Jul 15;100(2):509-516.

5. O'Brien L, Kerrigan SW, Kaw G, Hogan M, Penadés J, Litt D, et al. Multiple mechanisms for the activation of human platelet aggregation by Staphylococcus aureus: roles for the clumping factors ClfA and ClfB, the serine-aspartate repeat protein SdrE and protein A. Mol. Microbiol. 2002 May;44(4):1033-1044.

6. Erickson PR, Herzberg MC. A collagen-like immunodeterminant on the surface of Streptococcus sanguis induces platelet aggregation. J. Immunol. 1987 May 15;138(10):3360-3366.

7. Moreillon P, Que YA, Bayer AS. Pathogenesis of streptococcal and staphylococcal endocarditis. Infect. Dis. Clin. North Am. 2002 Jun;16(2):297-318.

8. Mylonakis E, Calderwood SB. Infective endocarditis in adults. N. Engl. J. Med. 2001 Nov 1;345(18):1318-1330.

9. Italiano JE Jr, Shivdasani RA. Megakaryocytes and beyond: the birth of platelets. J. Thromb. Haemost. 2003 Jun;1(6):1174-1182.

10. Ruggeri ZM. Platelets in atherothrombosis. Nat. Med. 2002 Nov;8(11):1227-1234.

11. Furie B, Furie BC. Thrombus formation in vivo. J. Clin. Invest. 2005 Dec;115(12):3355-3362.

12. Jennings LK, Fox JE, Edwards HH, Phillips DR. Changes in the cytoskeletal structure of human platelets following thrombin activation. J. Biol. Chem. 1981 Jul 10;256(13):6927-6932.

13. Shattil SJ, Newman PJ. Integrins: dynamic scaffolds for adhesion and signaling in platelets. Blood. 2004 Sep 15;104(6):1606-1615.

14. Reed GL, Fitzgerald ML, Polgár J. Molecular mechanisms of platelet exocytosis: insights into the “secrete” life of thrombocytes. Blood. 2000 Nov 15;96(10):3334 -3342.

15. Brass LF, Stalker TJ, Zhu L, Lu B, Woulfe DS, Prevost N. Boundary events: contact-dependent and contact-facilitated signaling between platelets. Semin. Thromb. Hemost. 2004 Aug;30(4):399-410.

16. Kulkarni S, Dopheide SM, Yap CL, Ravanat C, Freund M, Mangin P, et al. A revised model of platelet aggregation. J. Clin. Invest. 2000 Mar;105(6):783-791.

17. Hellem AJ. The adhesiveness of human blood platelets in vitro. Scand. J. Clin. Lab. Invest. 1960;12(Suppl):1-117.

18. Gaarder A, JONSEN J, LALAND S, HELLEM A, OWREN PA. Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets. Nature. 1961 Nov 11;192:531-532.

19. Cattaneo M. The P2 receptors and congenital platelet function defects. Semin. Thromb. Hemost. 2005 Apr;31(2):168-173.

20. Jin J, Daniel JL, Kunapuli SP. Molecular basis for ADP-induced platelet activation. II. The P2Y1 receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. J. Biol. Chem. 1998 Jan 23;273(4):2030-2034.

21. Kunapuli SP, Dorsam RT, Kim S, Quinton TM. Platelet purinergic receptors. Curr Opin Pharmacol. 2003 Apr;3(2):175-180.

22. Daniel JL, Dangelmaier C, Jin J, Kim YB, Kunapuli SP. Role of intracellular signaling events in ADP-induced platelet aggregation. Thromb. Haemost. 1999 Oct;82(4):1322-1326.

23. Marcus AJ, Broekman MJ, Drosopoulos JHF, Olson KE, Islam N, Pinsky DJ, et al. Role of CD39 (NTPDase-1) in thromboregulation, cerebroprotection, and cardioprotection. Semin. Thromb. Hemost. 2005 Apr;31(2):234-246.

24. Born GV, Cross MJ. The aggregation of blood platelets. J. Physiol. (Lond.). 1963 Aug;168:178-195.

25. Paul BZ, Jin J, Kunapuli SP. Molecular mechanism of thromboxane A(2)-induced platelet aggregation. Essential role for p2t(ac) and alpha(2a) receptors. J. Biol. Chem. 1999 Oct 8;274(41):29108-29114.

26. Cristalli G, Volpini R, Vittori S, Camaioni E, Monopoli A, Conti A, et al. 2-Alkynyl derivatives of adenosine-5'-N-ethyluronamide: selective A2 adenosine receptor agonists with potent inhibitory activity on platelet aggregation. J. Med. Chem. 1994 May 27;37(11):1720-1726.

27. Cristalli G, Vittori S, Thompson RD, Padgett WL, Shi D, Daly JW, et al. Inhibition of platelet aggregation by adenosine receptor agonists. Naunyn Schmiedebergs Arch. Pharmacol. 1994 Jun;349(6):644-650.

28. Springthorpe B, Bailey A, Barton P, Birkinshaw TN, Bonnert RV, Brown RC, et al. From ATP to AZD6140: the discovery of an orally active reversible P2Y12 receptor antagonist for the prevention of thrombosis. Bioorg. Med. Chem. Lett. 2007 Nov 1;17(21):6013-6018.

29. Hechler B, Vigne P, Léon C, Breittmayer JP, Gachet C, Frelin C. ATP derivatives are antagonists of the P2Y1 receptor: similarities to the platelet ADP receptor. Mol. Pharmacol. 1998 Apr;53(4):727-733.

30. MacKenzie AB, Mahaut-Smith MP, Sage SO. Activation of receptor-operated cation channels via P2X1 not P2T purinoceptors in human platelets. J. Biol. Chem. 1996 Feb 9;271(6):2879-2881.

31. Oury C, Toth-Zsamboki E, Thys C, Tytgat J, Vermylen J, Hoylaerts MF. The ATP-gated P2X1 ion channel acts as a positive regulator of platelet responses to collagen. Thromb. Haemost. 2001 Nov;86(5):1264-1271.

32. Samuelsson B, Goldyne M, Granström E, Hamberg M, Hammarström S, Malmsten C. Prostaglandins and thromboxanes. Annu. Rev. Biochem. 1978;47:997-1029.

33. Hung DT, Wong YH, Vu TK, Coughlin SR. The cloned platelet thrombin receptor couples to at least two distinct effectors to stimulate phosphoinositide hydrolysis and inhibit adenylyl cyclase. J. Biol. Chem. 1992 Oct 15;267(29):20831-20834.

34. Kim SO, Lim CT, Lam SC, Hall SE, Komiotis D, Venton DL, et al. Purification of the human blood platelet thromboxane A2/prostaglandin H2 receptor protein. Biochem. Pharmacol. 1992 Jan 22;43(2):313-322.

35. Ushikubi F, Nakajima M, Hirata M, Okuma M, Fujiwara M, Narumiya S. Purification of the thromboxane A2/prostaglandin H2 receptor from human blood platelets. J. Biol. Chem. 1989 Oct 5;264(28):16496-16501.

36. Djellas Y, Manganello JM, Antonakis K, Le Breton GC. Identification of Galpha13 as one of the G-proteins that couple to human platelet thromboxane A2 receptors. J. Biol. Chem. 1999 May 14;274(20):14325-14330.

37. Knezevic I, Borg C, Le Breton GC. Identification of Gq as one of the G-proteins which copurify with human platelet thromboxane A2/prostaglandin H2 receptors. J. Biol. Chem. 1993 Dec 5;268(34):26011-26017.

38. Offermanns S, Laugwitz KL, Spicher K, Schultz G. G proteins of the G12 family are activated via thromboxane A2 and thrombin receptors in human platelets. Proc. Natl. Acad. Sci. U.S.A. 1994 Jan 18;91(2):504-508.

39. Gabbeta J, Yang X, Kowalska MA, Sun L, Dhanasekaran N, Rao AK. Platelet signal transduction defect with Galpha subunit dysfunction and diminished Galphaq in a patient with abnormal platelet responses. Proc. Natl. Acad. Sci. U.S.A. 1997 Aug 5;94(16):8750-8755.

40. Offermanns S, Toombs CF, Hu YH, Simon MI. Defective platelet activation in G alpha(q)-deficient mice. Nature. 1997 Sep 11;389(6647):183-186.

41. Kerrigan SW, Cox D. Platelet-bacterial interactions. Cell. Mol. Life Sci. 2010 Feb;67(4):513-523.

42. Yeaman MR, Tang YQ, Shen AJ, Bayer AS, Selsted ME. Purification and in vitro activities of rabbit platelet microbicidal proteins. Infect. Immun. 1997 Mar;65(3):1023-1031.

43. Yeaman MR, Bayer AS. Antimicrobial peptides from platelets. Drug Resist. Updat. 1999 Apr;2(2):116-126.

44. Yeaman MR. The role of platelets in antimicrobial host defense. Clin. Infect. Dis. 1997 Nov;25(5):951-968; quiz 969-970.

45. Yeaman MR, Sullam PM, Dazin PF, Bayer AS. Platelet microbicidal protein alone and in combination with antibiotics reduces Staphylococcus aureus adherence to platelets in vitro. Infect. Immun. 1994 Aug;62(8):3416-3423.

46. Tang Y-Q, Yeaman MR, Selsted ME. Antimicrobial peptides from human platelets. Infect. Immun. 2002 Dec;70(12):6524-6533.

47. Cole AM, Ganz T, Liese AM, Burdick MD, Liu L, Strieter RM. Cutting edge: IFN-inducible ELR- CXC chemokines display defensin-like antimicrobial activity. J. Immunol. 2001 Jul 15;167(2):623-627.

48. Krijgsveld J, Zaat SA, Meeldijk J, van Veelen PA, Fang G, Poolman B, et al. Thrombocidins, microbicidal proteins from human blood platelets, are C-terminal deletion products of CXC chemokines. J. Biol. Chem. 2000 Jul 7;275(27):20374-20381.

49. Bayer AS, Cheng D, Yeaman MR, Corey GR, McClelland RS, Harrel LJ, et al. In vitro resistance to thrombin-induced platelet microbicidal protein among clinical bacteremic isolates of Staphylococcus aureus correlates with an endovascular infectious source. Antimicrob. Agents Chemother. 1998 Dec;42(12):3169-3172.

50. Fowler VG Jr, McIntyre LM, Yeaman MR, Peterson GE, Barth Reller L, Corey GR, et al. In vitro resistance to thrombin-induced platelet microbicidal protein in isolates of Staphylococcus aureus from endocarditis patients correlates with an intravascular device source. J. Infect. Dis. 2000 Oct;182(4):1251-1254.

51. Youssefian T, Drouin A, Massé J-M, Guichard J, Cramer EM. Host defense role of platelets: engulfment of HIV and Staphylococcus aureus occurs in a specific subcellular compartment and is enhanced by platelet activation. Blood. 2002 Jun 1;99(11):4021-4029.

52. Bhakdi S, Muhly M, Mannhardt U, Hugo F, Klapettek K, Mueller-Eckhardt C, et al. Staphylococcal alpha toxin promotes blood coagulation via attack on human platelets. J. Exp. Med. 1988 Aug 1;168(2):527-542.

53. Erickson PR, Herzberg MC. Purification and partial characterization of a 65-kDa platelet aggregation-associated protein antigen from the surface of Streptococcus sanguis. J. Biol. Chem. 1990 Aug 25;265(24):14080-14087.

54. Herzberg MC. Platelet-streptococcal interactions in endocarditis. Crit. Rev. Oral Biol. Med. 1996;7(3):222-236.

55. Herzberg MC, Brintzenhofe KL, Clawson CC. Aggregation of human platelets and adhesion of Streptococcus sanguis. Infect. Immun. 1983 Mar;39(3):1457-1469.

56. Herzberg MC, Gong K, MacFarlane GD, Erickson PR, Soberay AH, Krebsbach PH, et al. Phenotypic characterization of Streptococcus sanguis virulence factors associated with bacterial endocarditis. Infect. Immun. 1990 Feb;58(2):515-522.

57. Kerrigan SW, Jakubovics NS, Keane C, Maguire P, Wynne K, Jenkinson HF, et al. Role of Streptococcus gordonii surface proteins SspA/SspB and Hsa in platelet function. Infect. Immun. 2007 Dec;75(12):5740-5747.

58. Pampolina C, McNicol A. Streptococcus sanguis-induced platelet activation involves two waves of tyrosine phosphorylation mediated by FcgammaRIIA and alphaIIbbeta3. Thromb. Haemost. 2005 May;93(5):932-939.

59. Siauw C, Kobsar A, Dornieden C, Beyrich C, Schinke B, Schubert-Unkmeir A, et al. Group B streptococcus isolates from septic patients and healthy carriers differentially activate platelet signaling cascades. Thromb. Haemost. 2006 May;95(5):836-849.

60. Plummer C, Wu H, Kerrigan SW, Meade G, Cox D, Ian Douglas CW. A serine-rich glycoprotein of Streptococcus sanguis mediates adhesion to platelets via GPIb. Br. J. Haematol. 2005 Apr;129(1):101-109.

61. Bensing BA, López JA, Sullam PM. The Streptococcus gordonii surface proteins GspB and Hsa mediate binding to sialylated carbohydrate epitopes on the platelet membrane glycoprotein Ibalpha. Infect. Immun. 2004 Nov;72(11):6528-6537.

62. Takahashi Y, Yajima A, Cisar JO, Konishi K. Functional analysis of the Streptococcus gordonii DL1 sialic acid-binding adhesin and its essential role in bacterial binding to platelets. Infect. Immun. 2004 Jul;72(7):3876-3882.

63. Chia J-S, Lin Y-L, Lien H-T, Chen J-Y. Platelet aggregation induced by serotype polysaccharides from Streptococcus mutans. Infect. Immun. 2004 May;72(5):2605-2617.

64. Matsumoto-Nakano M, Tsuji M, Inagaki S, Fujita K, Nagayama K, Nomura R, et al. Contribution of cell surface protein antigen c of Streptococcus mutans to platelet aggregation. Oral Microbiol. Immunol. 2009 Oct;24(5):427-430.

65. Bensing BA, Rubens CE, Sullam PM. Genetic loci of Streptococcus mitis that mediate binding to human platelets. Infect. Immun. 2001 Mar;69(3):1373-1380.

66. Pietrocola G, Schubert A, Visai L, Torti M, Fitzgerald JR, Foster TJ, et al. FbsA, a fibrinogen-binding protein from Streptococcus agalactiae, mediates platelet aggregation. Blood. 2005 Feb 1;105(3):1052-1059.

67. Kurpiewski GE, Forrester LJ, Campbell BJ, Barrett JT. Platelet aggregation by Streptococcus pyogenes. Infect. Immun. 1983 Feb;39(2):704-708.

68. Shannon O, Hertzén E, Norrby-Teglund A, Mörgelin M, Sjöbring U, Björck L. Severe streptococcal infection is associated with M protein-induced platelet activation and thrombus formation. Mol. Microbiol. 2007 Sep;65(5):1147-1157.

69. Loughman A, Fitzgerald JR, Brennan MP, Higgins J, Downer R, Cox D, et al. Roles for fibrinogen, immunoglobulin and complement in platelet activation promoted by Staphylococcus aureus clumping factor A. Mol. Microbiol. 2005 Aug;57(3):804-818.

70. Fitzgerald JR, Loughman A, Keane F, Brennan M, Knobel M, Higgins J, et al. Fibronectin-binding proteins of Staphylococcus aureus mediate activation of human platelets via fibrinogen and fibronectin bridges to integrin GPIIb/IIIa and IgG binding to the FcgammaRIIa receptor. Mol. Microbiol. 2006 Jan;59(1):212-230.

71. Pawar P, Shin PK, Mousa SA, Ross JM, Konstantopoulos K. Fluid shear regulates the kinetics and receptor specificity of Staphylococcus aureus binding to activated platelets. J. Immunol. 2004 Jul 15;173(2):1258-1265.

72. George NPE, Wei Q, Shin PK, Konstantopoulos K, Ross JM. Staphylococcus aureus adhesion via Spa, ClfA, and SdrCDE to immobilized platelets demonstrates shear-dependent behavior. Arterioscler. Thromb. Vasc. Biol. 2006 Oct;26(10):2394-2400.

73. Shannon O, Flock J-I. Extracellular fibrinogen binding protein, Efb, from Staphylococcus aureus binds to platelets and inhibits platelet aggregation. Thromb. Haemost. 2004 Apr;91(4):779-789.

74. Siboo IR, Chambers HF, Sullam PM. Role of SraP, a Serine-Rich Surface Protein of Staphylococcus aureus, in binding to human platelets. Infect. Immun. 2005 Apr;73(4):2273-2280.

75. Usui Y, Ohshima Y, Ichiman Y, Ohtomo T, Suganuma M, Yoshida K. Platelet aggregation induced by strains of various species of coagulase-negative staphylococci. Microbiol. Immunol. 1991;35(1):15-26.

76. Lourbakos A, Yuan YP, Jenkins AL, Travis J, Andrade-Gordon P, Santulli R, et al. Activation of protease-activated receptors by gingipains from Porphyromonas gingivalis leads to platelet aggregation: a new trait in microbial pathogenicity. Blood. 2001 Jun 15;97(12):3790-3797.

77. Takii R, Kadowaki T, Baba A, Tsukuba T, Yamamoto K. A functional virulence complex composed of gingipains, adhesins, and lipopolysaccharide shows high affinity to host cells and matrix proteins and

escapes recognition by host immune systems. Infect. Immun. 2005 Feb;73(2):883-893.

78. Naito M, Sakai E, Shi Y, Ideguchi H, Shoji M, Ohara N, et al. Porphyromonas gingivalis-induced platelet aggregation in plasma depends on Hgp44 adhesin but not Rgp proteinase. Mol. Microbiol. 2006 Jan;59(1):152-167.

79. Fowler VG Jr, Miro JM, Hoen B, Cabell CH, Abrutyn E, Rubinstein E, et al. Staphylococcus aureus endocarditis: a consequence of medical progress. JAMA. 2005 Jun 22;293(24):3012-3021.

80. Fitzgerald JR, Foster TJ, Cox D. The interaction of bacterial pathogens with platelets. Nat. Rev. Microbiol. 2006 Jun;4(6):445-457.

81. Massey RC, Kantzanou MN, Fowler T, Day NP, Schofield K, Wann ER, et al. Fibronectin-binding protein A of Staphylococcus aureus has multiple, substituting, binding regions that mediate adherence to fibronectin and invasion of endothelial cells. Cell. Microbiol. 2001 Dec;3(12):839-851.

82. Jenkinson HF, Lamont RJ. Oral microbial communities in sickness and in health. Trends Microbiol. 2005 Dec;13(12):589-595.

83. Douglas CW, Heath J, Hampton KK, Preston FE. Identity of viridans streptococci isolated from cases of infective endocarditis. J. Med. Microbiol. 1993 Sep;39(3):179-182.

84. Bensing BA, Gibson BW, Sullam PM. The Streptococcus gordonii platelet binding protein GspB undergoes glycosylation independently of export. J. Bacteriol. 2004 Feb;186(3):638-645.

85. Takahashi Y, Ruhl S, Yoon J-W, Sandberg AL, Cisar JO. Adhesion of viridans group streptococci to sialic acid-, galactose- and N-acetylgalactosamine-containing receptors. Oral Microbiol. Immunol. 2002 Aug;17(4):257-262.

86. Nobbs AH, Shearer BH, Drobni M, Jepson MA, Jenkinson HF. Adherence and internalization of Streptococcus gordonii by epithelial cells involves beta1 integrin recognition by SspA and SspB (antigen I/II family) polypeptides. Cell. Microbiol. 2007 Jan;9(1):65-83.

87. Herzberg MC, MacFarlane GD, Gong K, Armstrong NN, Witt AR, Erickson PR, et al. The platelet interactivity phenotype of Streptococcus sanguis influences the course of experimental endocarditis. Infect. Immun. 1992 Nov;60(11):4809-4818.

88. Erickson PR, Herzberg MC, Tierney G. Cross-reactive immunodeterminants on Streptococcus sanguis and collagen. Predicting a structural motif of platelet-interactive domains. J. Biol. Chem. 1992 May 15;267(14):10018-10023.

89. Herzberg MC, Nobbs A, Tao L, Kilic A, Beckman E, Khammanivong A, et al. Oral streptococci and cardiovascular disease: searching for the platelet aggregation-associated protein gene and mechanisms of Streptococcus sanguis-induced thrombosis. J. Periodontol. 2005 Nov;76(11 Suppl):2101-2105.

90. Herzberg MC, Krishnan LK, MacFarlane GD. Involvement of alpha 2-adrenoreceptors and G proteins in the modulation of platelet secretion in response to Streptococcus sanguis. Crit. Rev. Oral Biol. Med. 1993;4(3-4):435-442.

91. Herzberg MC, Brintzenhofe KL. ADP-like platelet aggregation activity generated by viridans streptococci incubated with exogenous ATP. Infect. Immun. 1983 Apr;40(1):120-125.

92. MacFarlane GD, Sampson DE, Clawson DJ, Clawson CC, Kelly KL, Herzberg MC. Evidence for an ecto-ATPase on the cell wall of Streptococcus sanguis. Oral Microbiol. Immunol. 1994 Jun;9(3):180-185.

93. Cossart P, Jonquières R. Sortase, a universal target for therapeutic agents against gram-positive bacteria? Proc. Natl. Acad. Sci. U.S.A. 2000 May 9;97(10):5013-5015.

94. Zimmermann H. 5’-Nucleotidase: molecular structure and functional aspects. Biochem. J. 1992 Jul 15;285 (Pt 2):345-365.

95. Zimmermann H. Extracellular metabolism of ATP and other nucleotides. Naunyn Schmiedebergs Arch. Pharmacol. 2000 Nov;362(4-5):299-309.

96. Vogel M, Kowalewski H, Zimmermann H, Hooper NM, Turner AJ. Soluble low-Km 5’-nucleotidase from electric-ray (Torpedo marmorata) electric organ and bovine cerebral cortex is derived from the glycosyl-phosphatidylinositol-anchored ectoenzyme by phospholipase C cleavage. Biochem. J. 1992 Jun 15;284 (Pt 3):621-624.

97. Sharom FJ, McNeil GL, Glover JR, Seier S. Modulation of the cleavage of glycosylphosphatidylinositol-anchored proteins by specific bacterial phospholipases. Biochem. Cell Biol. 1996;74(5):701-713.

98. Martínez-Martínez A, Muñoz-Delgado E, Campoy FJ, Flores-Flores C, Rodríguez-López JN, Fini C, et al. The ecto-5’-nucleotidase subunits in

dimers are not linked by disulfide bridges but by non-covalent bonds. Biochim. Biophys. Acta. 2000 May 23;1478(2):300-308.

99. Volknandt W, Vogel M, Pevsner J, Misumi Y, Ikehara Y, Zimmermann H. 5'-nucleotidase from the electric ray electric lobe. Primary structure and relation to mammalian and procaryotic enzymes. Eur. J. Biochem. 1991 Dec 18;202(3):855-861.

100. Knöfel T, Sträter N. X-ray structure of the Escherichia coli periplasmic 5'-nucleotidase containing a dimetal catalytic site. Nat. Struct. Biol. 1999 May;6(5):448-453.

101. Koonin EV, Mushegian AR, Tatusov RL, Altschul SF, Bryant SH, Bork P, et al. Eukaryotic translation elongation factor 1 gamma contains a glutathione transferase domain--study of a diverse, ancient protein superfamily using motif search and structural modeling. Protein Sci. 1994 Nov;3(11):2045-2054.

102. Plesner L. Ecto-ATPases: identities and functions. Int. Rev. Cytol. 1995;158:141-214.

103. Naito Y, Lowenstein JM. 5'-Nucleotidase from rat heart membranes. Inhibition by adenine nucleotides and related compounds. Biochem. J. 1985 Mar 15;226(3):645-651.

104. Schnurr M, Then F, Galambos P, Scholz C, Siegmund B, Endres S, et al. Extracellular ATP and TNF-alpha synergize in the activation and maturation of human dendritic cells. J. Immunol. 2000 Oct 15;165(8):4704-4709.

105. Langston HP, Ke Y, Gewirtz AT, Dombrowski KE, Kapp JA. Secretion of IL-2 and IFN-gamma, but not IL-4, by antigen-specific T cells requires extracellular ATP. J. Immunol. 2003 Mar 15;170(6):2962-2970.

106. Thiel M, Caldwell CC, Sitkovsky MV. The critical role of adenosine A2A receptors in downregulation of inflammation and immunity in the pathogenesis of infectious diseases. Microbes Infect. 2003 May;5(6):515-526.

107. Thammavongsa V, Kern JW, Missiakas DM, Schneewind O. Staphylococcus aureus synthesizes adenosine to escape host immune responses. J. Exp. Med. 2009 Oct 26;206(11):2417-2427.

108. Eltzschig HK, Ibla JC, Furuta GT, Leonard MO, Jacobson KA, Enjyoji K, et al. Coordinated adenine nucleotide phosphohydrolysis and nucleoside signaling in posthypoxic endothelium: role of ectonucleotidases and adenosine A2B receptors. J. Exp. Med. 2003 Sep 1;198(5):783-796.

109. Goding JW, Grobben B, Slegers H. Physiological and pathophysiological functions of the ecto-nucleotide pyrophosphatase/phosphodiesterase family. Biochim. Biophys. Acta. 2003 May 20;1638(1):1-19.

110. Deussen A, Bading B, Kelm M, Schrader J. Formation and salvage of adenosine by macrovascular endothelial cells. Am. J. Physiol. 1993 Mar;264(3 Pt 2):H692-700.

111. Németh ZH, Csóka B, Wilmanski J, Xu D, Lu Q, Ledent C, et al. Adenosine A2A receptor inactivation increases survival in polymicrobial sepsis. J. Immunol. 2006 May 1;176(9):5616-5626.

112. Haskó G, Pacher P. A2A receptors in inflammation and injury: lessons learned from transgenic animals. J. Leukoc. Biol. 2008 Mar;83(3):447-455.

113. Khoa ND, Montesinos MC, Reiss AB, Delano D, Awadallah N, Cronstein BN. Inflammatory cytokines regulate function and expression of adenosine A(2A) receptors in human monocytic THP-1 cells. J. Immunol. 2001 Oct 1;167(7):4026-4032.

114. Panther E, Idzko M, Herouy Y, Rheinen H, Gebicke-Haerter PJ, Mrowietz U, et al. Expression and function of adenosine receptors in human dendritic cells. FASEB J. 2001 Sep;15(11):1963-1970.

115. Thompson LF, Ruedi JM, Glass A, Low MG, Lucas AH. Antibodies to 5'-nucleotidase (CD73), a glycosyl-phosphatidylinositol-anchored protein, cause human peripheral blood T cells to proliferate. J. Immunol. 1989 Sep 15;143(6):1815-1821.

116. Resta R, Yamashita Y, Thompson LF. Ecto-enzyme and signaling functions of lymphocyte CD73. Immunol. Rev. 1998 Feb;161:95-109.

117. Spychala J. Tumor-promoting functions of adenosine. Pharmacol. Ther. 2000 Sep;87(2-3):161-173.

118. Abraham J, Mansour C, Veledar E, Khan B, Lerakis S. Staphylococcus aureus bacteremia and endocarditis: the Grady Memorial Hospital experience with methicillin-sensitive S aureus and methicillin-resistant S aureus bacteremia. Am. Heart J. 2004 Mar;147(3):536-539.

119. Bouza E, Menasalvas A, Muñoz P, Vasallo FJ, del Mar Moreno M, García Fernández MA. Infective endocarditis--a prospective study at the end of the twentieth century: new predisposing conditions, new etiologic agents, and still a high mortality. Medicine (Baltimore). 2001 Sep;80(5):298-307.

120. Gould K, Ramirez-Ronda CH, Holmes RK, Sanford JP. Adherence of bacteria to heart valves in vitro. J. Clin. Invest. 1975 Dec;56(6):1364-1370.

121. Durack DT, Beeson PB. Experimental bacterial endocarditis. I. Colonization of a sterile vegetation. Br J Exp Pathol. 1972 Feb;53(1):44-49.

122. Durack DT. Experimental bacterial endocarditis. IV. Structure and evolution of very early lesions. J. Pathol. 1975 Feb;115(2):81-89.

123. Patti JM, Allen BL, McGavin MJ, Höök M. MSCRAMM-mediated adherence of microorganisms to host tissues. Annu. Rev. Microbiol. 1994;48:585-617.

124. Gong K, Wen DY, Ouyang T, Rao AT, Herzberg MC. Platelet receptors for the Streptococcus sanguis adhesin and aggregation-associated antigens are distinguished by anti-idiotypical monoclonal antibodies. Infect. Immun. 1995 Sep;63(9):3628-3633.

125. Benn M, Hagelskjaer LH, Tvede M. Infective endocarditis, 1984 through 1993: a clinical and microbiological survey. J. Intern. Med. 1997 Jul;242(1):15-22.

126. Hogevik H, Olaison L, Andersson R, Lindberg J, Alestig K. Epidemiologic aspects of infective endocarditis in an urban population. A 5-year prospective study. Medicine (Baltimore). 1995 Nov;74(6):324-339.

127. Hemler ME, Elices MJ, Parker C, Takada Y. Structure of the integrin VLA-4 and its cell-cell and cell-matrix adhesion functions. Immunol. Rev. 1990 Apr;114:45-65.

128. Veltrop MH, Thompson J, Beekhuizen H. Monocytes augment bacterial species- and strain-dependent induction of tissue factor activity in bacterium-infected human vascular endothelial cells. Infect. Immun. 2001 May;69(5):2797-2807.

129. Durack DT, Beeson PB. Experimental bacterial endocarditis. II. Survival of a bacteria in endocardial vegetations. Br J Exp Pathol. 1972 Feb;53(1):50-53.

130. Veltrop MH, Beekhuizen H, Thompson J. Bacterial species- and strain-dependent induction of tissue factor in human vascular endothelial cells. Infect. Immun. 1999 Nov;67(11):6130-6138.

131. Bayer AS, Sullam PM, Ramos M, Li C, Cheung AL, Yeaman MR. Staphylococcus aureus induces platelet aggregation via a fibrinogen-dependent mechanism which is independent of principal platelet

glycoprotein IIb/IIIa fibrinogen-binding domains. Infect. Immun. 1995 Sep;63(9):3634-3641.

132. Bancsi MJ, Veltrop MH, Bertina RM, Thompson J. Influence of monocytes and antibiotic treatment on tissue factor activity of endocardial vegetations in rabbits infected with Streptococcus sanguis. Infect Immun. 1996 Feb;64(2):448-451.

133. Veltrop MHAM, Bancsi MJLMF, Bertina RM, Thompson J. Role of Monocytes in Experimental Staphylococcus aureus Endocarditis. Infect. Immun. 2000 Aug 1;68(8):4818-4821.

134. Sullam PM, Frank U, Yeaman MR, Täuber MG, Bayer AS, Chambers HF. Effect of thrombocytopenia on the early course of streptococcal endocarditis. J. Infect. Dis. 1993 Oct;168(4):910-914.

135. Cheung AL, Koomey JM, Butler CA, Projan SJ, Fischetti VA. Regulation of exoprotein expression in Staphylococcus aureus by a locus (sar) distinct from agr. Proc. Natl. Acad. Sci. U.S.A. 1992 Jul 15;89(14):6462-6466.

136. Novick RP, Ross HF, Projan SJ, Kornblum J, Kreiswirth B, Moghazeh S. Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. EMBO J. 1993 Oct;12(10):3967-3975.

137. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. J. Clin. Periodontol. 1998 Feb;25(2):134-144.

138. Charo IF, Feinman RD, Detwiler TC. Interrelations of platelet aggregation and secretion. J. Clin. Invest. 1977 Oct;60(4):866-873.

139. Soberay AH, Herzberg MC, Rudney JD, Nieuwenhuis HK, Sixma JJ, Seligsohn U. Responses of platelets to strains of streptococcus sanguis: findings in healthy subjects, Bernard-Soulier, Glanzmann's, and collagen-unresponsive patients. Thromb. Haemost. 1987 Apr 7;57(2):222-225.

140. Woulfe D, Yang J, Brass L. ADP and platelets: the end of the beginning. J. Clin. Invest. 2001 Jun;107(12):1503-1505.

141. Huang EM, Detwiler TC. Characteristics of the synergistic actions of platelet agonists. Blood. 1981 Apr;57(4):685-691.

142. Sambrook J, Maniatis T, Fritsch E. Molecular cloning□: a laboratory manual. New York: Cold Spring Harbor Laboratory Press.; 1989.

143. Zhang Y, Lei Y, Khammanivong A, Herzberg MC. Identification of a novel two-component system in Streptococcus gordonii V288 involved in biofilm formation. Infect. Immun. 2004 Jun;72(6):3489-3494.

144. Macrina FL, Evans RP, Tobian JA, Hartley DL, Clewell DB, Jones KR. Novel shuttle plasmid vehicles for Escherichia-Streptococcus transgeneric cloning. Gene. 1983 Nov;25(1):145-150.

145. Nobbs AH, Zhang Y, Khammanivong A, Herzberg MC. Streptococcus gordonii Hsa environmentally constrains competitive binding by Streptococcus sanguinis to saliva-coated hydroxyapatite. J. Bacteriol. 2007 Apr;189(8):3106-3114.

146. Dunny GM, Lee LN, LeBlanc DJ. Improved electroporation and cloning vector system for gram-positive bacteria. Appl. Environ. Microbiol. 1991 Apr;57(4):1194-1201.

147. Xu P, Alves JM, Kitten T, Brown A, Chen Z, Ozaki LS, et al. Genome of the opportunistic pathogen Streptococcus sanguinis. J. Bacteriol. 2007 Apr;189(8):3166-3175.

148. Garen A, Levinthal C. A fine-structure genetic and chemical study of the enzyme alkaline phosphatase of E. coli. I. Purification and characterization of alkaline phosphatase. Biochim. Biophys. Acta. 1960 Mar 11;38:470-483.

149. Bengis-Garber C. Membrane-bound 5’-nucleotidase in marine luminous bacteria: biochemical and immunological properties. Can. J. Microbiol. 1985 Jun;31(6):543-548.

150. Itami H, Sakai Y, Shimamoto T, Hama H, Tsuda M, Tsuchiya T. Purification and characterization of membrane-bound 5’-nucleotidase of Vibrio parahaemolyticus. J. Biochem. 1989 May;105(5):785-789.

151. Sullam PM, Valone FH, Mills J. Mechanisms of platelet aggregation by viridans group streptococci. Infect. Immun. 1987 Aug;55(8):1743-1750.

152. Rolf MG, Mahaut-Smith MP. Effects of enhanced P2X1 receptor Ca2+ influx on functional responses in human platelets. Thromb. Haemost. 2002 Sep;88(3):495-502.

153. Jin J, Kunapuli SP. Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. Proceedings of the National Academy of Sciences of the United States of America. 1998 Jul 7;95(14):8070 -8074.

154. Baurand A, Raboisson P, Freund M, Léon C, Cazenave J-P, Bourguignon J-J, et al. Inhibition of platelet function by administration of MRS2179, a P2Y1 receptor antagonist. European Journal of Pharmacology. 2001 Feb 2;412(3):213-221.

155. Srinivasan S, Mir F, Huang J-S, Khasawneh FT, Lam SC-T, Le Breton GC. The P2Y12 Antagonists, 2-Methylthioadenosine 5′-Monophosphate Triethylammonium Salt and Cangrelor (ARC69931MX), Can Inhibit Human Platelet Aggregation through a Gi-independent Increase in cAMP Levels. Journal of Biological Chemistry. 2009 Jun 12;284(24):16108 -16117.

156. Dyszkiewicz-Korpanty A, Olteanu H, Frenkel EP, Sarode R. Clopidogrel anti-platelet effect: an evaluation by optical aggregometry, impedance aggregometry, and the platelet function analyzer (PFA-100). Platelets. 2007 Nov;18(7):491-496.

157. Hechler B, Magnenat S, Zighetti ML, Kassack MU, Ullmann H, Cazenave J-P, et al. Inhibition of platelet functions and thrombosis through selective or nonselective inhibition of the platelet P2 receptors with increasing doses of NF449 [4,4’,4’’,4’’’-(carbonylbis(imino-5,1,3-benzenetriylbis-(carbonylimino)))tetrakis-benzene-1,3-disulfonic acid octasodium salt]. J. Pharmacol. Exp. Ther. 2005 Jul;314(1):232-243.

158. Jacobson KA, Nikodijevic O, Padgett WL, Gallo-Rodriguez C, Maillard M, Daly JW. 8-(3-Chlorostyryl)caffeine (CSC) is a selective A2-adenosine antagonist in vitro and in vivo. FEBS Letters. 1993 May 24;323(1-2):141-144.

159. Turner NA, Moake JL, McIntire LV. Blockade of adenosine diphosphate receptors P2Y12 and P2Y1 is required to inhibit platelet aggregation in whole blood under flow. Blood. 2001 Dec 1;98(12):3340 -3345.

160. Kim S, Jin J, Kunapuli SP. Akt activation in platelets depends on Gi signaling pathways. J. Biol. Chem. 2004 Feb 6;279(6):4186-4195.

161. Lindup WE, Orme MC. Clinical pharmacology: plasma protein binding of drugs. Br Med J (Clin Res Ed). 1981 Jan 17;282(6259):212-214.

162. Boyer JL, Romero-Avila T, Schachter JB, Harden TK. Identification of competitive antagonists of the P2Y1 receptor. Mol. Pharmacol. 1996 Nov;50(5):1323-1329.

163. Weber AA, Reimann S, Schrör K. Specific inhibition of ADP-induced platelet aggregation by clopidogrel in vitro. Br. J. Pharmacol. 1999 Jan;126(2):415-420.

164. Nylander S, Mattsson C, Ramström S, Lindahl TL. Synergistic action between inhibition of P2Y12/P2Y1 and P2Y12/thrombin in ADP- and thrombin-induced human platelet activation. Br. J. Pharmacol. 2004 Aug;142(8):1325-1331.

165. Erickson PR, Herzberg MC. The Streptococcus sanguis platelet aggregation-associated protein. Identification and characterization of the minimal platelet-interactive domain. J. Biol. Chem. 1993 Jan 25;268(3):1646-1649.

166. Dorsam RT, Kunapuli SP. Central role of the P2Y12 receptor in platelet activation. J. Clin. Invest. 2004 Feb;113(3):340-345.

167. Dangelmaier C, Jin J, Smith JB, Kunapuli SP. Potentiation of thromboxane A2-induced platelet secretion by Gi signaling through the phosphoinositide-3 kinase pathway. Thromb. Haemost. 2001 Feb;85(2):341-348.

168. Kauffenstein G, Bergmeier W, Eckly A, Ohlmann P, Léon C, Cazenave JP, et al. The P2Y(12) receptor induces platelet aggregation through weak activation of the alpha(IIb)beta(3) integrin--a phosphoinositide 3-kinase-dependent mechanism. FEBS Lett. 2001 Sep 14;505(2):281-290.

169. Woulfe D, Jiang H, Mortensen R, Yang J, Brass LF. Activation of Rap1B by G(i) family members in platelets. J. Biol. Chem. 2002 Jun 28;277(26):23382-23390.

170. Weber AA, Hohlfeld T, Schrör K. cAMP is an important messenger for ADP-induced platelet aggregation. Platelets. 1999 Jul;10(4):238-241.

171. Nesbitt WS, Giuliano S, Kulkarni S, Dopheide SM, Harper IS, Jackson SP. Intercellular calcium communication regulates platelet aggregation and thrombus growth. J. Cell Biol. 2003 Mar 31;160(7):1151-1161.

172. Miller OV, Gorman RR. Modulation of platelet cyclic nucleotide content by PGE1 and the prostaglandin endoperoxide PGG2. J Cyclic Nucleotide Res. 1976;2(2):79-87.

173. Jin J, Quinton TM, Zhang J, Rittenhouse SE, Kunapuli SP. Adenosine diphosphate (ADP)-induced thromboxane A(2) generation in human platelets requires coordinated signaling through integrin alpha(IIb)beta(3) and ADP receptors. Blood. 2002 Jan 1;99(1):193-198.

174. Schnurr M, Then F, Galambos P, Scholz C, Siegmund B, Endres S, et al. Extracellular ATP and TNF-α Synergize in the Activation and Maturation of Human Dendritic Cells. The Journal of Immunology. 2000 Oct 15;165(8):4704 -4709.

175. Imai M, Goepfert C, Kaczmarek E, Robson SC. CD39 modulates IL-1 release from activated endothelial cells. Biochem. Biophys. Res. Commun. 2000 Apr 2;270(1):272-278.

176. Borsellino G, Kleinewietfeld M, Di Mitri D, Sternjak A, Diamantini A, Giometto R, et al. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. Blood. 2007 Aug 15;110(4):1225-1232.

177. Deaglio S, Dwyer KM, Gao W, Friedman D, Usheva A, Erat A, et al. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. J. Exp. Med. 2007 Jun 11;204(6):1257-1265.

178. Schlievert PM, Gahr PJ, Assimacopoulos AP, Dinges MM, Stoehr JA, Harmala JW, et al. Aggregation and binding substances enhance pathogenicity in rabbit models of Enterococcus faecalis endocarditis. Infect. Immun. 1998 Jan;66(1):218-223.

179. Schlievert PM, Chuang-Smith ON, Peterson ML, Cook LCC, Dunny GM. Enterococcus faecalis Endocarditis Severity in Rabbits Is Reduced by IgG Fabs Interfering with Aggregation Substance. PLoS ONE. 2010 Oct 4;5(10):e13194.

180. Neu HC. The 5'-nucleotidases and cyclic phosphodiesterases (3'-nucleotidases) of the Enterobacteriaceae. J. Bacteriol. 1968 May;95(5):1732-1737.

APPENDIX

ECTO-5'-NUCLEOTIDASE: A COMMON MODULATOR FOR STREPTOCOCCAL-PLATELET INTERACTION

The ecto-nucleotidase activity on *S. sanguinis* 133-79 modulates bacterial-platelet interactions. By searching the genome of another *S. sanguinis* strain, SK36, four putative cell surface ecto-nucleotidases proteins were identified. To identify their enzymatic activities, each of those genes was inactivated individually. Compared to its wild-type strain, the putative ecto-5'-nucleotidase (NT5E) deletion mutant (Δ*nt5e*), demonstrated significantly reduced cell surface hydrolytic activities for the adenine nucleotides, ATP, ADP and AMP. Moreover, in the absence of *nt5e*, platelets had a shorter lag time to the onset of aggregation. However, Δ*nt5e* had normal platelet adhesion ability. Since both ADP removal and adenosine generation inhibit platelet aggregation, these results suggested that the enzymatic activities of NT5E modulate platelet aggregation. The modulation of platelet-bacterial interactions by NT5E may be a common mechanism among *S. sanguinis* strains.

INTRODUCTION

Streptococcus sanguinis adhere to and activate human platelets to aggregate (54). In response to *S. sanguinis*, platelet activation is associated with secretion of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) from dense granules (90). The extracellular ADP is a potent platelet agonist and amplifies platelet aggregation induced by other pro-thrombotic agonists (138,141), whereas, the final product of hydrolysis of adenine nucleotides, adenosine, is a platelet aggregation antagonist (26). We showed that cell surface ecto-5'-nucleotidase (NT5E) activities modulate bacterial-platelet interactions (chapter 3). To determine whether this is a common mechanism, we characterized the NT5E activities on *S. sanguinis* strain SK36, and its role in platelet aggregation.

By searching the whole genome of *S. sanguinis* SK36, four putative proteins possessing LPXTG motifs were identified that could hydrolyze extracellular nucleotides (Table 1). To characterize their enzymatic activities, we inactivated the four genes separately and examined the whole cell enzymatic activities for adenosine nucleotides hydrolysis. Compared to the wild-type strain, one of the mutants, the putative ecto-5'-nucleotidase deletion mutant (Δ*nt5e*) had significantly decreased cell-surface ATPase, ADPase and AMPase activities. Moreover, in the absence of *nt5e*, the lag time to the onset of platelet aggregation is shortened. However, Δ*nt5e* had normal platelet adhesion ability. Since cell-surface NT5E activities are widely distributed among *S. sanguinis*

strains (chapter 3), the modulation of NT5E in platelet-bacterial interactions might be a common mechanism among *S. sanguinis* strains, and perhaps other bacterial pathogens, such as staphylococci and enterococci, which cause bloodborne infections.

MATERIALS AND METHODS

Bacterial strains and culture conditions. In *S. sanguinis* SK36, we constructed a 5'-nucleotidase deletion mutant (Δ*nt5e*), extracellular nuclease deletion mutant, (Δ*nucH*), cyclo nucleotide phosphodiesterase deletion mutant (Δ*cnp*), and DNA repair ATPase deletion mutant (Δ*rad3*). Mutants were constructed by allelic exchange as described in chapter 3. Primers used in this study were listed in table 2. *S. sanguinis* SK36 and the isogenic mutants were routinely grown in Todd Hewitt Broth (TH broth, Difco; Sparks, MD) or on TH agar plates at 37℃ in 5% CO_2. When required, antibiotics were added to the medium at the indicated concentrations: erythromycin (Em), 10 µg ml^{-1}.

Nucleotidase activity.

AMPase activity. Streptococcal cells in stationary phase were harvested after 16 h in THB. Harvested cells were washed twice with 30 mM Tris•HCl buffer (pH 7.4) containing 0.25 mM ethylenediamine tetraacetic acid (EDTA) and 30 mM

Table 1. *S. sanguinis* SK36 cell-surface proteins potentially possess nucleotidase activities

Proteins, putative	Accession No.[a]	Substrate specificity
5'-nucleotidase	A3CN82	5'-nucleotides with preference for adenine nucleotides
extracellular nuclease	A3CPM7	Ribonucleic acids to nucleoside monophosphates or diphosphates
cyclo-nucleotide phosphodiesterase	A3CKJ7	Nucleoside 2',3'-cyclic phosphate to nucleoside 3'-phosphate
DNA repair ATPase	A3CKA3	adenosine triphosphate to adenosine diphosphate

[a]Available from GenBank.

Table 2. Primers used in this study

Primer	Sequence (5' to 3')
SK36A1F	ACGTCCGGTACCTTCTTGATTCCTGCCAATCC
SK36A1R	ACGTCCGAATTCCAGCTAATGCGGCTTCCTAC
SK36A2F	ACGTCCGGATCCAGCATGGTATAGCCGTCACC
SK36A2R	ACGTCCGAGCTCATGAGAACGGACAACCCTTG
SK36B1F	ACGTCCGGTACCAACATCAGCAGGGTCAATCC
SK36B1R	ACGTCCGAATTCCCCAACCGTCTCAATCAACT
SK36B2F	ACGTCCGGATCCCACTGCCATTCTCGTCTTGA
SK36B2R	ACGTCCGAGCTCACTCAGCTGCCTGATTTGGT
SK36C1F	ACGTCCGGTACCGGTATCAGCGAAATGCCAGT
SK36C1R	ACGTCCGAATTCGGCAAGGAGATTGATCCAAA
SK36C2F	ACGTCCGGATCCCATGCCTGCTGTCTCGATTA
SK36C2R	ACGTCCGAGCTCTCGAGAAGGCCAAGAAAGAA
SK36D1F	ACGTCCGGTACCAGGATTTGACAACGGTCAGG
SK36D1R	ACGTCCGAATTCATCCTCAGCTGCAGCAATTT
SK36D2F	ACGTCCGGATCCGAAAACAGCGCTTGAAAAGG
SK36D2R	ACGTCCGAGCTCCGCATCTGTCACCGTCTTTA

[a] All primers were designed as part of this study.

[b] Underlined letters indicate restriction enzyme site.

sodium chloride (NaCl), followed by washing with 50 mM Tris•HCl (pH 7.4) containing 130 mM sodium chloride (NaCl) and 5 mM magnesium dichloride ($MgCl_2$), and resuspended to 2 X 10^9 cells per ml. To each 2 ml microcentrifuge tube, 0.5 ml bacterial suspension was mixed with AMP added to a final concentration of 10, 50 or 100 µM and incubated at 37°C for 30 minutes. After incubation, cells were centrifuged at 10,000 x g for 5 min, and 50 µl of the supernatant from each tube was transferred into a 96-well plates (Corning, NY). The AMPase activity was measured as the amount of inorganic phosphate (Pi) released into the supernatants using the QuantiChrom Phosphate Assay Kit DIPI-500 (Bioassay systems, Hayward, CA). The results were expressed as nM of Pi produced/min per 10^6 cells,

Phosphate hydrolase activity. After removal of the culture medium, the streptococcal cells were washed twice with 50 mM Tris•HCl (pH 8.0) buffer containing 150 mM NaCl, 5 mM $CaCl_2$, and 5 mM $MgCl_2$. The cells were then incubated with 10, 50 and 100 µl ADP or ATP at 37℃ for 30 minutes. The enzymatic activity was measured as described above.

Platelet aggregometry. Strains of *S. sanguinis* were tested for the ability to induce platelet aggregation with fresh PRP obtained from a single donor as described previously (55). A single donor was used to eliminate variability in platelet response between donors (139). Each bacterial strain (50 µl suspension

containing 4 X 10^9 cells/ml) was incubated with 450 µl of PRP (4 X 10^8 cells/ml). PRP aggregation was performed at 37℃ with controlled stirring in a recording aggregometer (model 660, Chronolog Corp., Havertown, PA), and the lag time or delay to onset (minutes) was measured.

Platelet bacterial adhesion assay (PBAA). All procedures were performed as described previously (55). In brief, platelets from outdated PRP (Memorial Blood Center, St. Paul, MN) were washed with PBS and fixed with 10% formalin. Washed platelets and washed streptococcal cells were incubated together or alone (controls) in microwells; the small clusters of adhering platelets and bacteria were separated from non-interacting particles by centrifugation. The sedimentation of adhering mixtures relative to controls was quantitated by the following formula: percent adhesion = 100 X {1 – [mixture $A_{620\ nm}$/(bacterium $A_{620\ nm}$ + washed-platelet $A_{620\ nm}$)/2]}. Based on previous studies of the variability of the method, only adhesion scores of ≥ 20% were considered positive.

Gene sequences. The genome of *S. sanguinis* SK36 is available at http://www.ncbi.nlm.nih.gov/genome?Db=genome&Cmd=ShowDetailView&TermToSearch=20499.

Statistical analysis. Descriptive statistics, including the mean, standard deviation (n = 2) and standard error (n = 3), were calculated. Statistical analysis

of data was performed using the Student's *t*-test, or two-way analysis of variance (two-way ANOVA) with GraphPad Prism 5 (GraphPad Software, La Jolla, CA). The level of significance $\alpha = 0.05$ was considered to be statistically significant.

RESULTS

Identification of the gene possessing NT5E activities on *S. sanguinis*. We measured *S. sanguinis* whole cell enzymatic activities. *S. sanguinis* SK36 wild-type (wt) hydrolyzed the adenine nucleotides ATP (Figure 1A), ADP (Figure 1B) and AMP (Figure 1C) in a dose-dependent manner. In the absence of *nt5e*, the ATPase activity on Δ*nt5e* was only significantly reduced with 10 - 100 μM ATP, which is consistent with the concomitant presence of an ecto-ATPase on the cell surface of *S. sanguinis* as we reported (92). Compared to wt, the cell-surface ADPase and AMPase activities were almost fully abrogated. In contrast, inactivation of *nucH, cnp* or *rad3*, which has putative nucleotidase activity, did not affect the whole cell enzymatic activities of *S. sanguinis* SK36. Collectively, there results strongly suggest that *nt5e* has ATPase, ADPase and AMPase activities.

Inhibition of platelet aggregation by NT5E. Using platelet aggregometry, we compared the aggregation lag-time and magnitude in response to wt and Δ*nt5e*, respectively. In response to wt, platelets from a single donor aggregated in

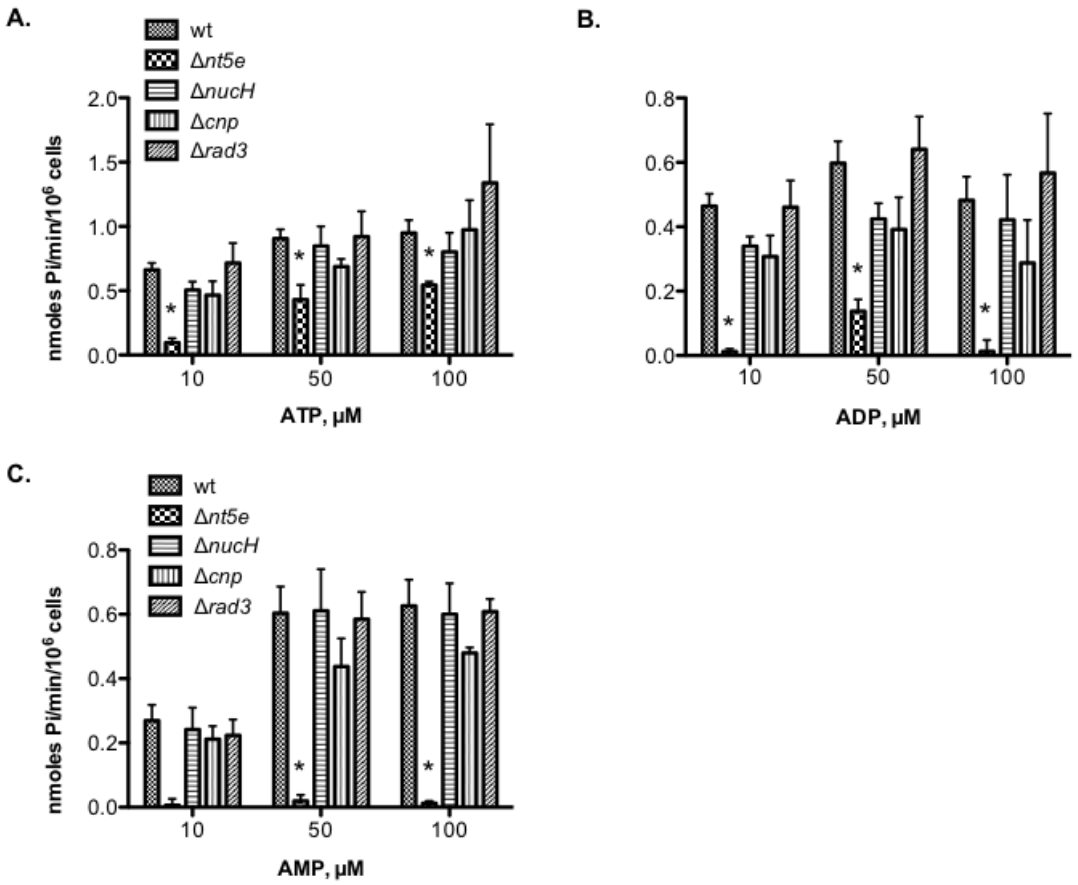


Figure 1. *nt5e* possesses NT5E activity on *S. sanguinis* SK36 whole cell. NT5E activity was measured by the release of inorganic phosphate (Pi) from adenine nucleotides. (A), (B), and (C) were showed as enzyme velocity vs. concentration of ATP, ADP and AMP substrates. The results were represented as Mean ± SE, n = 3.

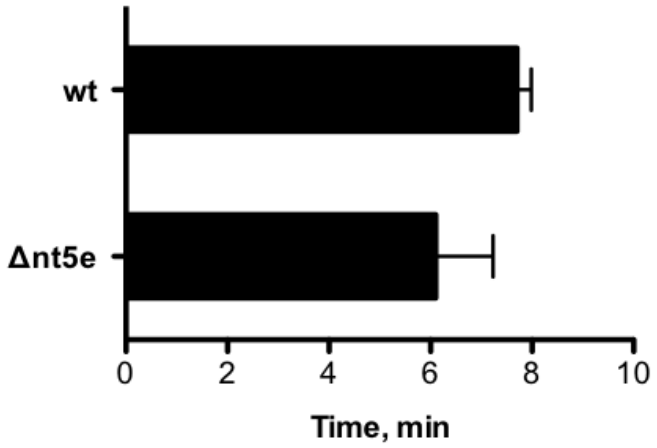


Figure 2. NT5E affects platelet aggregation lag time.

Response leading to aggregation was recorded as the mean lag-time to onset of aggregation ± SD, N=2.

approximately 8 min (Figure 2A; n = 2). The aggregation response of Δ*nt5e* showed a lag-time of approximately 6 min, which was shorter than the wt strain (Figure 2A; n = 2). The magnitude of aggregation, however, was not affected. Therefore, the *S. sanguinis* SK36 NT5E prolonged the lag time to the onset of platelet aggregation.

Adhesion of platelets and bacteria. The percent adhesion to platelets was similar for wt (60.1 ± 1.9%), Δ*nt5e* (56.5 ± 1.4%) strains.

DISCUSSION

We have previously showed ecto-5'-nucleotidase activities on the cell wall of *S. sanguinis* 133-79 modulate platelet aggregation in vitro (chapter 3). We sought to further determine whether this is a common mechanism among viridans streptococci. Here, we characterized the NT5E activities on another *S. sanguinis* strain, SK36. A putative *nt5e* was identified and characterized to have NT5E activities on the bacterial cell surface. Similar to *S. sanguinis* 133-79, *S. sanguinis* SK36 NT5E can hydrolyze extracellular ATP to ADP, AMP and adenosine (Figure 1). Since ADP and adenosine each contributes to the platelet aggregation response, we hypothesized that the *S. sanguinis* NT5E modulates platelet aggregation.

Expectedly, an *nt5e* deletion mutant of *S. sanguinis* SK36 showed a shorter platelet aggregation lag time but similar magnitude of aggregation when compared to the wild-type strain (Figure 2). Inactivation of *nt5e* gene, however, did not affect the ability of *S. sanguinis* SK36 to adhere to human platelets.

These results suggested that the lag time is in part a response to the NT5E activity on the surface of cells of many species. In conclusion, we provided evidence that NT5E might be a common modulator of *S. sanguinis*-induced platelet aggregation in vitro, which may be applicable to other bacterial pathogens, such as staphylococci and enterococci, which can be bloodborne pathogens.