Rapid screening method for detecting highly pathogenic *Streptococcus intermedius* strains carrying a mutation in the *lacR* gene.

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24 **Key words:** *Streptococcus intermedius*, Intermedilysin, Screening, MsgA, NanA, LacR.

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26 **One sentence summary:** Rapid screening method for highly pathogenic *S. intermedius*

27 strains based on the ratio of activities of MsgA and NanA resulting from *lacR*

28 mutations.

ABSTRACT

Streptococcus intermedius is a member of the normal human commensal flora and secretes a human-specific cytolysin intermedilysin (ILY) as a major virulence factor. Expression of *ily* is repressed by LacR and loss-of-function mutations of LacR are observed in many ILY high-producing strains isolated from deep-seated abscesses, suggesting that high ILY production is necessary for increased virulence. However, because ILY exhibits no β-hemolysis on animal blood agar plates, differentiating ILY high- and low-producing strains using conventional laboratory methods is not possible. Interestingly, *S. intermedius* also produces glycosidases, including MsgA and NanA, which exhibit *N*-acetyl-β-D-glucosaminidase and neuraminidase activities, respectively. Moreover, MsgA expression, but not NanA, is negatively regulated by LacR. Here we measured the activities of MsgA, NanA and ILY in strains isolated from clinical specimens and dental plaque to determine the correlation between these glycosidase activities and ILY hemolytic activity. Hemolytic activity showed a strong positive correlation with MsgA and a weak negative correlation with NanA activities. Therefore, we calculated the ratio of MsgA and NanA activity (M/N ratio). This value showed a

45 stronger positive correlation (r = 0.81) with ILY hemolytic activity and many strains

46 with high M/N ratios (> 2) were ILY high-producers with loss-of-function mutations in

47 LacR.

48

INTRODUCTION

Streptococcus intermedius is a facultatively anaerobic, opportunistic pathogen that belongs to the Anginosus group of streptococci (Whiley *et al.* 1990; Jensen *et al.* 2013). This pathogen is associated with oral infections, including periodontal disease and recurrent tonsillitis, and with deep-seated purulent infections such as brain and lung abscesses (Whiley *et al.* 1992; Jerng *et al.* 1997; Tanner *et al.* 1997; Gray 2005; Takayanagi *et al.* 2010; Weaver *et al.* 2010; Saito *et al.* 2012; Noguchi *et al.* 2015; Yost *et al.* 2015). *S. intermedius* produces a human-specific cytotoxin intermedilysin (ILY) of the cholesterol-dependent cytolysin (CDC) family, encoded by the *ily* gene (Nagamune *et al.* 1996). ILY is believed to be a crucial virulence factor for infectivity and toxicity to human cells, because knockout mutations of *ily* or inactivation of ILY using an anti-ILY antibody result in greatly decreased cytotoxicity (Sukeno *et al.* 2005). In contrast to other CDC family members, ILY does not use cholesterol as a primary binding receptor and can specifically recognize a glycosylphosphatidylinositol-linked human cell membrane protein, CD59 (Giddings *et al.* 2004). Therefore, *S. intermedius* is considered to be a strictly human-specific pathogen.

Two transcriptional repressors that can regulate *ily* expression have been reported thus far: catabolite control protein (CcpA) and lactose phosphotransferase system repressor (LacR) (Tomoyasu *et al.* 2010; Tomoyasu *et al.* 2013). LacR can repress transcription of the *ily* operon by binding to the *ily* promoter (Tomoyasu *et al.* 2013). Disruption of *lacR* in *S. intermedius* has been shown to cause constitutive overproduction of ILY and increased toxicity to the human hepatoma cell line HepG2 (Tomoyasu *et al.* 2013). Because a loss-of-function mutation in LacR is observed in almost all ILY high-producing strains isolated from deep-seated abscesses, high production of ILY seems to be necessary for increased virulence of this bacterium (Tomoyasu *et al.* 2013).

S. intermedius produces glycosidases such as MsgA and NanA (Takao *et al.* 2010; Imaki *et al.* 2014). MsgA has four glycosidase activities (β-D-galactosidase, β-D-fucosidase, *N*-acetyl-β-D-glucosaminidase, and *N*-acetyl-β-D-galactosaminidase) (Imaki *et al.*, 2014) which are stably conserved across all *S. intermedius* strains reported to date (Jensen *et al.* 2013; Imaki *et al.* 2014). Because *msgA* is localized in the *lac* operon, its expression, like that of *ily*, is regulated by LacR (Imaki *et al.* 2014). In

addition, *S. intermedius* is the only species among the Anginosus group of streptococci reported to exhibit neuraminidase activity. This glycosidase activity (NanA) is stably conserved in all *S. intermedius* strains (Jensen *et al.* 2013).

Because of the correlation between higher production of ILY and increased virulence of *S. intermedius* an accurate and rapid method for detecting ILY high-producing strains is required. However, since human-specific ILY does not produce β-hemolysis on animal blood agar plates (Nagamune *et al.* 1996), the detection of ILY high-producing strains and differentiation from ILY low-producing strains by conventional laboratory testing is not routinely possible. Therefore, we developed a rapid and simple method for discriminating these strain types by calculating the ratio between *N*-acetyl-β-D-glucosaminidase (MsgA) and neuraminidase (NanA) activities.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The bacterial strains and the positions of amino acid substitutions in LacR are listed in Table 1 (Takao *et al.* 2004; Tomoyasu *et al.* 2013). *S. intermedius* was cultured at 37 °C under anaerobic conditions (N_2:H_2:CO_2 = 85:10:5). Brain-heart infusion (BHI) broth (Becton-Dickinson, Palo Alto, CA, USA) and 3-(*N*-morpholino)propanesulfonic acid-buffered BHI (MOPS-BHI) broth were used as the culture media (Tomoyasu *et al.* 2010).

Nucleotide sequences of *lacR* from *S. intermedius* clinical isolates.

Fragments of the *lacR* gene from strains TK-I2, TK-I15, TK-I19, TK-I16, TK-I20, TU-C11, TU-C41, TU-C43, TU-C45, TU-C46, IW-I1 and IW-I2 were amplified by PCR and sequenced as previously described (Tomoyasu *et al.* 2013).

Measurement of *N*-acetyl-β-D-glucosaminidase and neuraminidase activities.

107 *S. intermedius* strains were pre-cultured in 10 mL BHI broth at 37 °C for 24 h.
108 Subsequently, 50 μL of pre-culture was inoculated in 5 mL MOPS–BHI broth and
109 cultured at 37 °C for 24 h under anaerobic conditions. The optical density at 600 nm
110 (OD_{600}) of the bacterial culture was measured for the hemolysis assay, and then the
111 culture supernatant and the cells were separated by centrifugation. The culture
112 supernatant was used for the hemolysis assay and the cell pellet was used for the
113 measurement of glycosidase activities as described below. The cell pellet was suspended
114 in 20 mM Tris-HCl buffer (pH 7.5) to OD_{600} = 1.0. Cell-associated glycosidase
115 activities were determined using chromogenic substrates. Assays were carried out in
116 96-well plates with a final volume of 100 μl/well. *N*-acetyl-*β*-D-glucosaminide activity
117 (MsgA activity) was determined using 10 μL of cell suspension in 70 mM sodium
118 citrate buffer (pH 5.5) containing 500 μM 4-nitrophenyl *N*-acetyl-*β*-D-glucosaminide.
119 Neuraminidase activity (NanA activity) was determined using 50 μL or 15 μL of cell
120 suspension in 70 mM sodium citrate buffer (pH 5.5) containing 200 μM
121 2-O-(*p*-Nitrophenyl)-*α*-D-N-acetylneuraminic acid. The 96-well plate was incubated in a
122 PST-100HL plate shaker thermostat (Biosan, Riga, Latvia) at 50°C for 1 h with shaking

123 at 1000 rpm. After the reaction, 100 µL of 0.5 M sodium carbonate buffer (pH 10.2) was
124 added to terminate the enzymatic reaction and development of the color. Subsequently,
125 the A_{405} of each well was measured in a Multiskan FC microplate photometer (Thermo
126 Fisher Scientific Inc., MA, USA). MsgA and NanA activities relative to these activities
127 observed with PC574 as a reference strain were calculated as follows: Relative activity
128 = [A_{405} of the sample after incubation – A_{405} of the sample without incubation/(A_{405} of
129 PC574 after incubation – A_{405} of PC574 without incubation]. The MsgA/NanA (M/N)
130 ratio was calculated as follows: M/N ratio = Relative activity of MsgA/Relative activity
131 of NanA.
132
133 **Hemolysis assay.**
134 Hemolysis was assayed as described previously (Tomoyasu *et al.* 2017). Relative
135 hemolytic activity = (dilution rate of culture supernatant sample giving 50% of
136 hemolysis/dilution rate of culture supernatant of reference strain UNS38 giving 50% of
137 hemolysis) × 10.
138

Statistics.

All experiments were performed in triplicate. Results were expressed as means ± standard deviations. Correlation between glycosidase activities or M/N ratios and hemolytic activities was evaluated by Pearson's product-moment correlation coefficient.

RESULTS AND DISCUSSION

Evidence has shown ILY expression to be a major virulence factor in *S. intermedius*. Furthermore, the majority of highly pathogenic *S. intermedius* strains isolated from deep-seated abscesses have a loss-of-function mutation in LacR which causes higher production of ILY than in the less pathogenic strains most frequently isolated from normal sites (Nagamune *et al*. 2000, Tomoyasu *et al*. 2017). Therefore, it is important to develop a rapid detection method for the presence of ILY high-producing strains. *S. intermedius* strains can be identified at the species level by rapid PCR methods that amplify *ily* or a ribosomal RNA coding region (Nagamune *et al.* 2000, Takao *et al.* 2004). Whereas ILY acts specifically on human cells, *S. intermedius* does not exhibit β-hemolysis on animal blood agar (Nagamune *et al.* 1996) and therefore it is impossible to discriminate the ILY high-producing strains from the ILY low-producing ones of this species by routine laboratory blood agar culture. Furthermore, the strategy of using nucleotide sequencing of *lacR* of all isolates to check for amino acid substitutions is not realistic, as this is costly and time-consuming. In addition, amino acid substitutions alone cannot be used to estimate LacR activity. For

160 example, a mutation from cysteine to tyrosine at position 135 (C135Y) in LacR is a
161 neutral mutation (Tomoyasu *et al.* 2013) and has been observed in 17/64 strains
162 including both ILY low- and high-producing strains used in this study (data not shown).
163 MsgA activity is increased by disruption of *lacR*, because *msgA* localizes in the *lac*
164 operon repressed by LacR (Imaki *et al.* 2014). Therefore, we examined the relationship
165 between MsgA activity and ILY mediated hemolytic activity within strains. MsgA and
166 hemolytic activities were assayed in 56 strains isolated from clinical specimens (11 of
167 which had a loss-of-function mutation in LacR), 7 strains isolated from dental plaque,
168 and the type strain (NCDO2227) (Table 1 and supplementary Table 1). MsgA has two
169 catalytic domains (LacZ and CH20); the LacZ domain confers β-D-galactosidase
170 activity and the CH20 domain confers *N*-acetyl-β-D-glucosaminidase activity (Imaki *et*
171 *al.* 2014). Because *N*-acetyl-β-D-glucosaminidase activity had a higher k_{cat}/K_m value and
172 higher thermostability than β-D-galactosidase (Imaki *et al.* 2014), we measured
173 *N*-acetyl-β-D-glucosaminidase activity as an index of MsgA activity at 50 °C. The
174 results obtained demonstrate that MsgA activities of strains showed a strong positive
175 correlation with hemolytic activities (Pearson's product-moment correlation coefficient;

176 r = 0.73) (Fig. 1a). Exceptions were only observed with three ILY high-producing
177 strains (NMH2, UNS32, and UNS45) which had a loss-of-function mutation in LacR
178 but showed lower MsgA activity than some ILY low-producing strains (supplementary
179 Table 1). We determined hemolytic activity of the strains that showed more than 10% of
180 the activity of UNS38 and thereby were classified as an ILY high-producing strains.
181 The majority of ILY low-producing strains have only weak hemolytic activity, these
182 activity levels were sufficiently low to preclude accurate determinations and these were
183 therefore scored as zero.

184 Previously, we observed approximately 50 % reduction in NanA activity for a strain
185 with disrupted *lacR* compared to a wild-type strain, although the reason for this is still
186 unknown (Imaki *et al.* 2014). Therefore, we examined the correlation between NanA
187 activity and hemolytic activity (Fig. 1b, supplementary Table 2). Because the optimal
188 temperature for NanA activity is 52°C (unpublished data), this activity could be
189 measured under the same conditions as for determining MsgA activity. A weak negative
190 correlation (r = -0.28) was observed between NanA and hemolytic activities for all

191 strains tested and with an increased negative correlation (r = -0.54) observed for the 16
192 ILY high-producing strains tested.

193 Because MsgA and NanA activities showed positive and negative correlations with
194 hemolytic activity respectively, we calculated the ratio between relative activity of
195 MsgA and NanA (M/N ratio) (Fig. 2). The M/N ratio was strongly correlated with
196 hemolytic activity (r = 0.81). Among 14 strains with a high M/N ratio (> 2), 12 strains
197 were high producers of ILY and 11 strains had loss-of-function mutations in LacR. No
198 strain that had a loss-of-function mutations in LacR had an M/N ratio < 2. ILY
199 high-producing strain TK-I2 had a R143C mutation in LacR. Because arginine residue
200 143 of *S. intermedius* LacR is well-conserved among the LacR homologs of
201 streptococci (data not shown), this mutation seems to cause reduction in, or inactivation
202 of LacR function. On the other hand, in strain HW7 a loss-of-function mutation in LacR
203 did not lead to overproduction of ILY (Tomoyasu *et al.* 2013).

204 Based on these results we conclude that determining the M/N ratio is an effective
205 index for discriminating between potentially clinically significant, highly toxigenic
206 (ILY high-producing) strains with *lacR* mutations and ILY low-producing strains.

207 Interestingly, some exceptions were observed: ILY high-producing strain UNS40 and
208 ILY low-producing strain JICC32122 both had wild-type LacR, but showed higher M/N
209 ratios. Strains F600, JICC33405, JICC674, and UNS42 exhibited ILY high-producing
210 phenotypes but had wild-type LacR and lower M/N ratios (< 2). These data suggest that
211 *ily* expression in these strains is activated by a mutation other than in the LacR gene.
212 Further elucidation of the mechanisms underlying ILY expression levels in *S.*
213 *intermedius* will contribute to a greater understanding of the variation in virulence
214 observed for strains of this human pathogen.

215 It has been shown that *S. intermedius* is more virulent in patients
216 immunocompromised due to aging, diabetes, cirrhosis and cancer than in
217 immunocompetent individuals (Murray *et al.* 1978; Jacobs *et al.* 1995; Jerng *et al.*
218 1997; Bert *et al.* 1998; Takayanagi *et al.* 2010; Noguchi *et al.* 2015). It is thought that
219 this is especially so for ILY high-producing strains which would pose a higher risk to
220 immunocompromised individuals (Tomoyasu *et al.* 2017). Therefore, an increasing
221 number of serious infections with ILY high-producing strains can be anticipated in
222 populations with increasing proportions of elderly people. Our screening method

adopting the ratio of two glycosidase activities can be carried out in virtually identical test formats making testing simpler and therefore less prone to experimental error. This screening method will be useful for predicting the existence of highly pathogenic strains in the normal flora and at the site of focal infections. An earlier diagnosis might help to prevent serious infections by highly pathogenic strains.

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238

239 **Figure legends**

240 **Fig 1** Correlation between MsgA activity and hemolytic activity (a). Vertical axis shows
241 relative MsgA activity (PC574 set as 1) and horizontal axis represents relative
242 hemolytic activity (UNS38 set as 10). Correlation between NanA activity and hemolytic
243 activity (b). Vertical axis shows relative NanA activity (PC574 set as 1) and horizontal
244 axis represents relative hemolytic activity. Relative activities of each strain are
245 represented as open circles. Relative hemolytic activity of ILY-low producing strains
246 (less than 10 % activity of UNS38) is plotted as 0 on the horizontal axis. Error bars
247 show standard deviations from three independent experiments.

248

249 **Fig 2** Correlation between M/N ratio and hemolytic activity. All cells were grown in
250 MOPS–BHI broth for 24 h at 37 °C. Left vertical axis shows M/N ratio (PC574 set as 1),
251 right vertical axis shows relative hemolytic activity (UNS38 set as 10), and strain name
252 is shown on the horizontal axis. Black bars show M/N ratio of strains that have a
253 loss-of-function mutation in LacR. Gray bar shows M/N ratio of strain TK-I2, which

has an uncharacterized mutation in LacR. White bars show M/N ratio of strains with wild-type LacR. The hemolytic activity of each strain is shown with red circles. The hemolytic activity of the strains without open circle is < 1.

257 **Table 1.** *S. intermedius* strains used for this study.

Strains	Isolation source	Mutation[a]
UNS38	Brain abscess	V21D
UNS46	Liver abscess	L48F
A4676a	Brain abscess	R37L
JICC 33616	Brain abscess	42Q_44Ldup
JICC 40138-2	Infective endocarditis	42Q_44Ldup
UNS32	Liver abscess	S117I
UNS45	Liver abscess	V30A
UNS35	Brain abscess	R50W
HW7	Brain abscess	S117N
JICC 32122	Brain abscess	–
UNS40	Liver abscess	–
JICC 1063	Liver abscess	V30A
TK-I2	unknown	R143C[b]
NMH2	Brain abscess	V21D
PC7466	Dental plaque	–
DP101	Dental abscess	–
JICC 32135	Empyema, mediastinitis	–
F600	Abdominal abscess	–
HW13	Abdominal Umbilical	–
DP102	Dental plaque	–
P58	Gingivitis	–
TK-I15	unknown	–
TK-I19	unknown	–
AC4720	Dental plaque	–
CDC415/87	Brain abscess	–
GN472	Dental plaque	–
TU-C11	dental abscess	–
2Q	Brain abscess	–
P88	Gingivitis	–

JICC 33405	Empyema, mediastinitis	–
JICC 689	Infective endocarditis	–
HW58	Brain abscess	–
TK-I16	unknown	–
JICC 32132	Brain abscess	–
JICC 32100	Septicemia	–
NMH8	Unknown	–
P101	Gingivitis	–
JICC 53299	Suppurative arthritis	–
WS100s	Bite wound, hand	–
AC5803	Dental plaque	–
TK-I20	Unknown	–
PC574	Dental plaque	–
P22	Gingivitis	–
JICC 33404	Pelvic abscess	–
NCDO2227	Type strain	–
JICC 33620	Brain abscess	–
TU-C41	dental abscess	–
JICC 674	Septicemia	–
JICC 32138	mediastinitis	–
AC800	Dental plaque	–
JICC 32151	Empyema, mediastinitis	–
JICC 33412	Subcutaneous abscess	–
P68	Gingivitis	–
F458s	Abdominal mass	–
UNS42	Liver abscess	–
HW69	Brain abscess	–
TU-C43	dental abscess	–
E691	Eye	–
JICC 33494	Brain abscess	–
JICC 33425	Subcutaneous abscess	–
TU-C46	dental abscess	–

IW-I2	unknown	–
TU-C45	dental abscess	–
IW-I1	unknown	–

[a]– No loss-of-function mutation in LacR. [b] Function of LacR R143C was not determined.

REFERENCES

Bert F, Bariou-Lancelin M, Lambert-Zechovsky N Clinical significance of bacteremia involving the "Streptococcus milleri" group: 51 cases and review. *Clin Infect Dis* 1998; **27**: 385-37.

Giddings KS, Zhao J, Sims PJ, *et al*. Human CD59 is a receptor for the cholesterol-dependent cytolysin intermedilysin. *Nat Struct Mol Biol* 2004; **11**: 1173-8.

Gray T. *Streptococcus anginosus* group: Clinical significance of an important group of pathogens. *Clinical Microbiology Newsletter* 2005; **27**: 155-9.

Imaki H, Tomoyasu T, Yamamoto N, *et al*. Identification and characterization of a novel secreted glycosidase with multiple glycosidase activities in *Streptococcus intermedius*. *J Bacteriol* 2014; **196**: 2817-26.

Jacobs JA, Pietersen HG, Stobberingh EE, Soeters PB *Streptococcus anginosus*, *Streptococcus constellatus* and *Streptococcus intermedius*. Clinical relevance, hemolytic and serologic characteristics. *Am J Clin Pathol* 1995; **104**: 547-53.

Jensen A, Hoshino T, Kilian M Taxonomy of the Anginosus group of the genus Streptococcus and description of *Streptococcus anginosus* subsp. *whileyi* subsp. nov. and *Streptococcus constellatus* subsp. *viborgensis* subsp. nov. *Int J Syst Evol Microbiol* 2013; **63**: 2506-19.

Jerng JS, Hsueh PR, Teng LJ, *et al*. Empyema thoracis and lung abscess caused by viridans streptococci. *Am J Respir Crit Care Med* 1997; **156**: 1508-14.

Murray HW, Gross KC, Masur H, *et al*. Serious infections caused by *Streptococcus milleri*. *Am J Med* 1978; **64**: 759-64.

Nagamune H, Ohnishi C, Katsuura A, *et al*. Intermedilysin, a novel cytotoxin specific for human cells secreted by *Streptococcus intermedius* UNS46 isolated from a human liver abscess. *Infect Immun* 1996; **64**: 3093-100.

Nagamune H, Whiley RA, Goto T, *et al*. Distribution of the intermedilysin gene among the anginosus group streptococci and correlation between intermedilysin production and deep-seated infection with *Streptococcus intermedius*. *J Clin Microbiol* 2000; **38**: 220-6.

Noguchi S, Yatera K, Kawanami T, *et al*. The clinical features of respiratory infections caused by the *Streptococcus anginosus* group. *BMC Pulm Med* 2015; **15**: 133.

292 Saito N, Hida A, Koide Y*, et al.* Culture-negative brain abscess with *Streptococcus*
293 *intermedius* infection with diagnosis established by direct nucleotide sequence
294 analysis of the 16s ribosomal RNA gene. *Internal medicine (Tokyo, Japan)* 2012;
295 **51**: 211-6.
296 Sukeno A, Nagamune H, Whiley RA, *et al*. Intermedilysin is essential for the invasion
297 of hepatoma HepG2 cells by *Streptococcus intermedius*. *Microbiol Immunol* 2005;
298 **49**: 681-94.
299 Takao A, Nagamune H, Maeda N Identification of the anginosus group within the genus
300 Streptococcus using polymerase chain reaction. *FEMS Microbiol Lett* 2004; **233**:
301 83-9.
302 Takao A, Nagamune H, Maeda N Sialidase of *Streptococcus intermedius*: a putative
303 virulence factor modifying sugar chains. *Microbiol Immunol* 2010; **54**: 584-595.
304 Takayanagi N, Kagiyama N, Ishiguro T, *et al*. Etiology and outcome of
305 community-acquired lung abscess. *Respiration; international review of thoracic*
306 *diseases* 2010; **80**: 98-105.
307 Tanner A, Maiden MF, Lee K, *et al*. Dental implant infections. *Clin Infect Dis* 1997; **25**
308 **Suppl 2**: S213-7.
309 Tomoyasu T, Yamasaki T, Chiba S, *et al*. Positive and negative control pathways by
310 blood components for intermedilysin production in *Streptococcus intermedius*.
311 *Infect Immun*.2017; **85**: e00379-17.
312 Tomoyasu T, Tabata A, Hiroshima R, *et al*. Role of catabolite control protein A in the
313 regulation of intermedilysin production by *Streptococcus intermedius*. *Infect Immun*
314 2010; **78**: 4012-21.
315 Tomoyasu T, Imaki H, Masuda S*, et al.* LacR mutations are frequently observed in
316 *Streptococcus intermedius* and are responsible for increased intermedilysin
317 production and virulence. *Infect Immun* 2013; **81**: 3276-86.
318 Weaver E, Nguyen X, Brooks MA Descending necrotising mediastinitis: two case
319 reports and review of the literature. *European respiratory review : an official*
320 *journal of the European Respiratory Society* 2010; **19**: 141-9.
321 Whiley RA, Fraser H, Hardie JM, *et al*. Phenotypic differentiation of *Streptococcus*
322 *intermedius*, *Streptococcus constellatus*, and *Streptococcus anginosus* strains within
323 the "Streptococcus milleri group". *J Clin Microbiol* 1990; **28**: 1497-501.
324 Whiley RA, Beighton D, Winstanley TG, *et al*. *Streptococcus intermedius*,

325 *Streptococcus constellatus*, and *Streptococcus anginosus* (the Streptococcus milleri
326 group): association with different body sites and clinical infections. *J Clin*
327 *Microbiol* 1992; **30**: 243-4.
328 Yost S, Duran-Pinedo AE, Teles R, *et al*. Functional signatures of oral dysbiosis during
329 periodontitis progression revealed by microbial metatranscriptome analysis.
330 *Genome medicine* 2015; **7**: 27.

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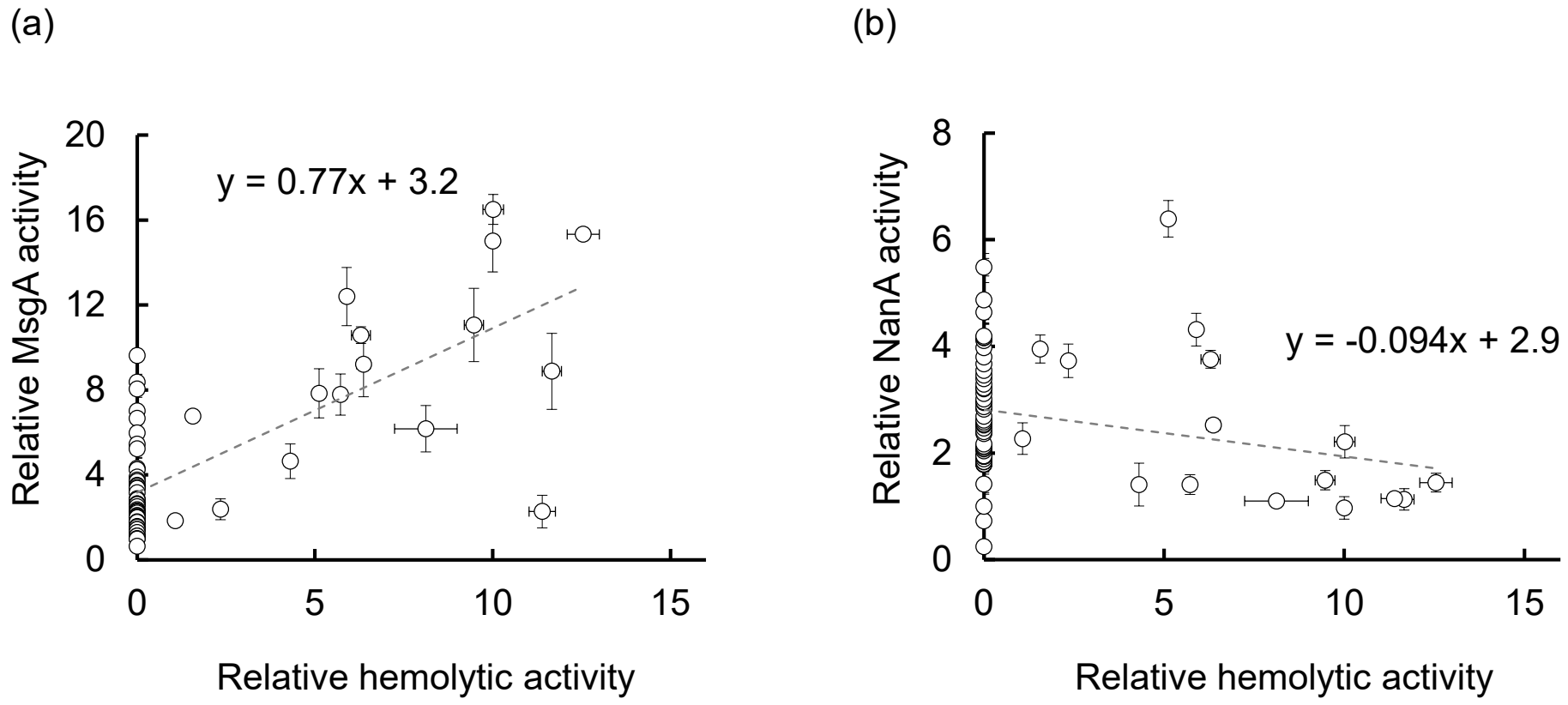
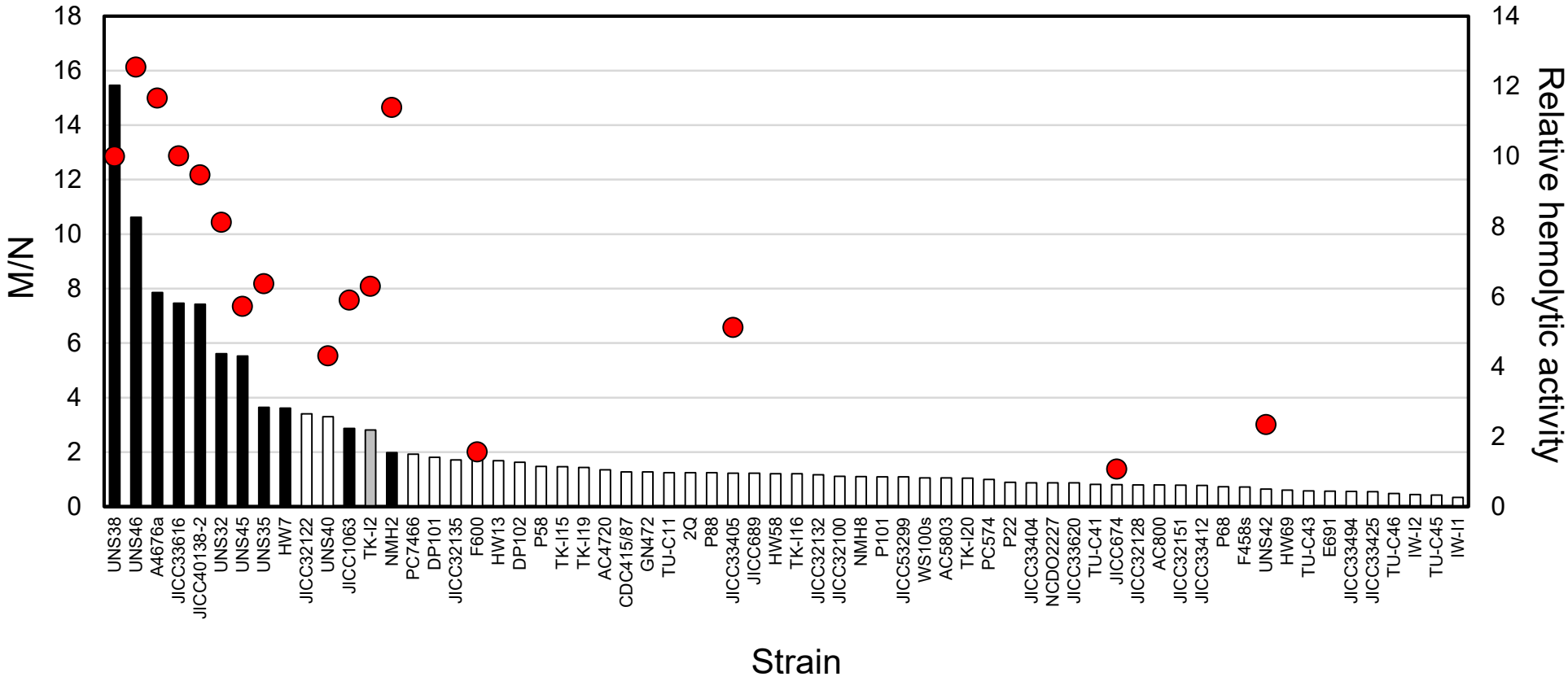


Tomoyasu *et al*. Fig. 1



Tomoyasu *et al*. Fig. 2