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Bacterial doubling time modulates the effects of opsonisation and available iron upon interactions between *Staphylococcus aureus* and human neutrophils

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Abstract

Staphylococcus aureus was grown exponentially at two doubling times (DT), one related to in vivo (DT 60 min) and one typical of laboratory conditions (DT 24 min), and under iron-poor and iron-rich conditions. Relative to the fast-grown phenotypes, both slow-grown phenotypes exhibited low surface hydrophobicity and low protein A expression, induced poorly in non-opsonised and opsonised chemiluminescence, and survived well in whole blood killing. In particular, slow-grown, iron-poor cocci demonstrated enhanced survival in whole blood killing which correlated with a significant reduction in their association with polymorphonuclear leukocytes, compared to the three other phenotypes; iron sufficiency increased the ability to stimulate polymorphonuclear leukocytes irrespective of opsonisation status. Staphylococcal DT may, by influencing surface hydrophobicity, modify interactions with immune system components.

Keywords: Polymorphonuclear leukocytes; Chemiluminescence; Killing; *Staphylococcus aureus*; Doubling time; Hydrophobicity; Opsonization; Iron

1. Introduction

Phagocytosis is the first line of defence against staphylococcal sepsis, entailing an intimate association between bacterial surface structures and polymorphonuclear leukocyte (PMN) membrane receptors. Some of these receptors are involved in non-specific interactions, e.g. surface hydrophobicity (SH), and under some circumstances the degree of bacterial SH influences the extent of the above association [1,2]. In turn, bacterial surface characteristics and particularly their doubling times (DT) reflect the nutritional restrictions that they encounter [3,4]; iron, for example, was determined to influence *Staphylococcus aureus* physiology long ago [5].

We decided to establish whether changes in *S. aureus* DT that could induce surface alterations could

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also influence PMN–staphylococcal interactions, in addition to the recognised effects of iron stress and opsonisation. In current literature, bacteria are grown usually in complex media and often into stationary phase or into exponential phase with a DT of 24–30 min. However, the use of complex media excludes the direct regulation of DT by, for example, chemostats. Also, these require the use of chemically defined media which cannot approximate the situation in vivo.

Here, we grew *S. aureus* in complex medium and, by changes in aeration, achieved relatively slow (DT 60 min) and relatively fast (DT 24 min) exponential DTs to reflect in vivo and laboratory conditions respectively [3,4]. Then, the influence of DT upon surface properties of *S. aureus* and its consequence for interactions with human PMN was determined.

2. Materials and methods

2.1. Growth conditions

Tryptone soy broth (TSB; Oxoid) was pre-treated with Chelex-100 resin (Bio-Rad), and selectively reconstituted and reinforced with cations and vitamins to give iron-rich and iron-poor TSB (TSB + Fe, 78.88 μM Fe; TSB − Fe, 1.97 μM Fe) [6]. 1 ml of overnight iron-poor *S. aureus* 2645 [7] was inoculated into pre-warmed 250 ml volumes of TSB + Fe and TSB − Fe in 1 litre flasks and grown at 37°C. Relatively slow exponential growth (DT 60 min) or relatively fast exponential growth (DT 24 min) was obtained by shaking at 150 or 600 rpm respectively in an orbital incubator (Gallenkamp). The resultant four phenotypes were harvested after three generations into exponential phase [8]. Growth was monitored by optical density determinations calibrated previously against a viable count standard curve.

2.2. Surface hydrophobicity

Cocci were harvested, washed, resuspended in 0.85% (w/v) NaCl to a final volume of 25 ml and E_{470} 20.0, and filtered onto water-washed 0.2 μm cellulose acetate membranes (Millipore). The contact angle (CA) made between the dried (3 h, 22°C) bacterial lawn and a 20 μl drop of 0.85% NaCl was measured [8].

2.3. Protein A

Cell-bound and soluble (supernatant) protein A (PrA) were assayed using sheep erythrocytes (Gibco) sensitised with dog anti-sheep erythrocytes (Miles), and incorporating PrA (salt-free; Sigma) standards [9].

2.4. Chemiluminescence

Chemiluminescence (CL) was by luminol enhancement [10]. Isolated PMN (MonoPoly Resolving Medium; Flow Labs) were resuspended to $\sim 1.0 \times 10^5 \pm 0.1$ ml^{-1} in Hanks' balanced salts solution, pH 7.4 (HBSS, Sigma) and the final density determined (model STKR; Coulter). Cocci grown as above were resuspended in HBSSCaMg (HBSS containing 0.95 mM $CaCl_2$, 0.41 mM $MgSO_4 \cdot 2H_2O$ and 0.49 mM $MgCl_2 \cdot 6H_2O$) to approximately 2×10^8 CFU ml^{-1}, and opsonised with fresh, autologous serum (20% final v/v, 15 min at 37°C). After, they were washed twice with GHBSSCaMg (HBSSCaMg containing 0.1% w/v gelatin), resuspended to $\sim 4 \times 10^7$ CFU ml^{-1} and sampled for counting chamber and viability count determinations to confirm the density. Then PMN and opsonised cocci were resuspended in luminol–GHBSSCaMg (enhancer) to give approximately 1 PMN:20 cfu and 0.01 μM luminol in a total volume of 4.5 ml [10]. GHBSSCaMg replaced serum in non-opsonised conditions and the control stimulant was phorbol 12-myristate 13-acetate (PMA, Sigma). Vials were prepared in duplicate. Enhancer alone or mixtures of enhancer plus cocci or PMN or PMA gave baseline corrections. Low levels of superoxide release were detected by counting out of coincidence (model LS 6800, Beckman).

2.5. Whole blood killing

Whole blood killing (WBK) [11] was performed simultaneously with CL. Whole blood and cocci from above were combined in an approximately 1 PMN:20 cfu ratio and rocked gently (37°C, 60 min). 30 (T30) and 60 (T60) min samples were vortexed

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vigorously, diluted appropriately and plated. Viability counts were expressed as a percentage of the original challenge. Smears were made at T30, stained (DiffQuik™) and examined to determine the numbers of cocci associated with the PMN.

3. Results and discussion

3.1. SH and PrA expression

The topical aspects unique to each phenotype were maintained post-harvest (Table 1), surface perturbation by exogenous chelators [12] being avoided by the use of Chelex-100 resin. *S. aureus* grown exponentially but relatively slowly (DT 60 min) was far less hydrophobic, particularly when iron-stressed, than when grown exponentially but relatively quickly (DT 24 min). A correlation was found as cell-bound PrA, a significant contributor to SH [13], was reduced in the slow-grown phenotypes, especially in the TSB − Fe cocci. Significantly, the latter had CA values ~ 5–6° less than those of human PMN (18–19° [2,14]), implying, from interfacial tension principles [2], that it would be the least easily phagocytosed of the phenotypes. In contrast, both fast-grown phenotypes exhibited a uniform, high SH that was indifferent to iron status. This was not reflected entirely by the cell-bound PrA trend, as the reduction in DT to 24 min permitted iron stress to disrupt PrA expression significantly. Perhaps in this type of CA assay, maximum SH values plateau once a threshold level of PrA expression is attained, as in the fast-grown TSB − Fe phenotype. For soluble (i.e. excreted) PrA, a slow DT allowed iron stress to reduce excretion as it was only detected in slow-grown TSB + Fe cocci. Neither fast-grown phenotype excreted detectable PrA, presumably this helped maintain their significantly elevated SH (surface charge experiments using Dowex-1 resin showed that all phenotypes were equally and highly negatively charged; data not shown).

3.2. CL and WBK

Non-opsonised CL directly examined the influence of SH upon *S. aureus*–PMN interactions. The slow-grown phenotypes with a DT of a magnitude found in vivo induced negligible CL bursts, particularly the TSB − Fe cocci (Fig. 1A). In contrast both fast-grown phenotypes with DTs more typical of exponential phase bacteria described in the current literature induced significant CL activity with the TSB − Fe cocci being less provocative. Non-opsonic assessments of phagocytic function therefore underlined the DT-induced topical variation that enabled each phenotype to stimulate PMN differently.

Opsonised CL involved specific and non-specific interactions due to antibody and complement respectively, and all phenotypes induced greater bursts (Fig. 1B). However, both slow-grown phenotypes again were less stimulatory than the fast-grown ones

Table 1
The expression of surface hydrophobicity (SH) and protein A (PrA; cell-bound, soluble i.e. supernatant) by slow-grown (DT 60 min) and fast-grown (DT 24 min) exponential phase iron-rich (TSB + Fe) and iron-poor (TSB − Fe) phenotypes of *S. aureus*

Phenotype	SH ° [a]			μg cell-bound PrA [b]			μg soluble PrA [b]
DT 60 min:							
TSB + Fe	21.78 ± 4.39	$P < 0.0001$ [c],	$P < 0.0001$ [d]	0.0690 ± 0.0120	$P = 0.5101$ [c],	$P < 0.0001$ [d]	0.0198
TSB − Fe	12.95 ± 2.71		$P < 0.0001$ [d]	0.0302 ± 0.0053		$P < 0.031$ [d]	ND
DT 24 min:							
TSB + Fe	58.88 ± 3.14	$P = 0.5268$ [c]		1.3789 ± 0.2433	$P < 0.0001$ [c]		ND
TSB − Fe	59.44 ± 3.61			0.1618 ± 0.0401			ND

[a] Mean ± S.D., 3 experiments, ≥ 10 CAs per phenotype.
[b] Mean ± S.D., relative to 10^8 cfu ml^{-1}, 3 experiments in triplicate.
[c] Comparison with TSB − Fe cocci; *t*-test.
[d] Comparisons of slow-grown TSB + Fe cocci with fast-grown TSB + Fe cocci, and of slow-grown TSB − Fe cocci with fast-grown TSB − Fe cocci; *t*-test.
ND, not detected.

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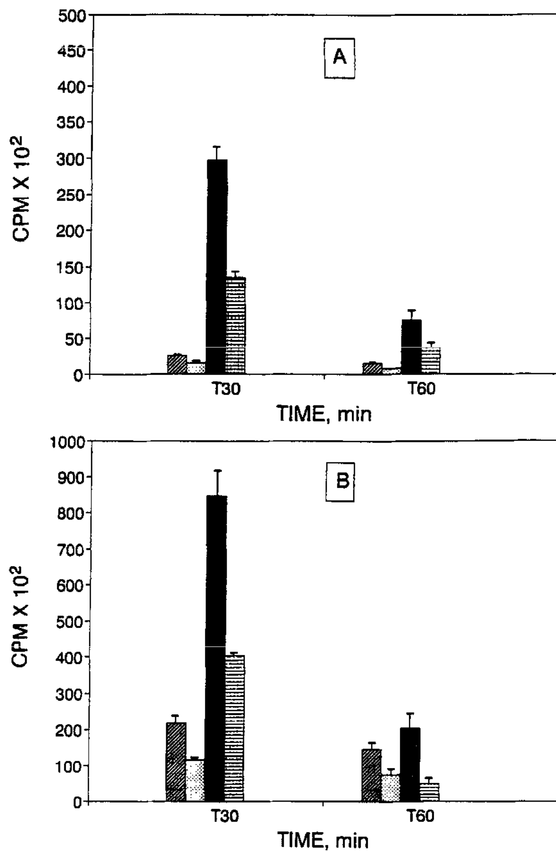

Fig. 1. The differing abilities of slow-grown (DT 60 min) and fast-grown (DT 24 min) exponential phase iron-rich (TSB + Fe) and iron-poor (TSB − Fe) phenotypes of *S. aureus* 2645 to stimulate PMN in luminol-enhanced CL: (A) non-opsonised, (B) opsonised. Isolated PMN and cocci were combined in a final ratio of ~ 1:20, with a final luminol concentration of 0.01 μM. Duplicate vials were read in a scintillation counter set to count out of coincidence. Baseline-corrected 30 min (T30) and 60 min (T60) trends are presented; mean ± S.D. of three experiments in duplicate, bars denote S.D. Columns indicate, from left to right: DT 60 min, TSB + Fe; DT 60 min, TSB − Fe; DT 24 min, TSB + Fe; DT 24 min, TSB − Fe.

which activated PMN most readily with accompanying very high CL bursts. The latter phenotypes, especially TSB + Fe cocci, also were most sensitive to killing by PMN (Fig. 2). Crucially, slow-grown TSB − Fe cocci were least potent in stimulating PMN in both assays despite opsonisation (Fig. 1B, Fig. 2). Also, the slow-grown TSB + Fe phenotype, although progressively killed in WBK, survived relatively well at e.g. T30, compared to its fast-grown counterpart.

Iron excess had increased the SH of only slow-grown TSB − Fe cocci but it improved the ability of both slow- and fast-grown TSB − Fe cocci to stimulate PMN. This may reflect differences between the physico-chemical and biological assays and generally the results above correlated positively with the SH data. Elsewhere, an enhanced PMN CL response was linked to an increased iron content of *S. aureus* [8,15] while an increase in iron content for stationary phase *S. aureus* grown in complex media (i.e. already iron-rich) with or without an iron supplement, resulted in an increased susceptibility to killing by H_2O_2 mechanisms [15,16].

In stained smears (WBK; T30 sampling), PMN challenged with the slow-grown, TSB − Fe phenotype had few associated cocci which correlated with their enhanced survival in WBK (Table 2, Fig. 3). PMN–*S. aureus* association was augmented significantly by excess iron or by a reduction in DT; the latter also abolished the iron-induced difference. Consequently, the slow-grown TSB + Fe phenotype

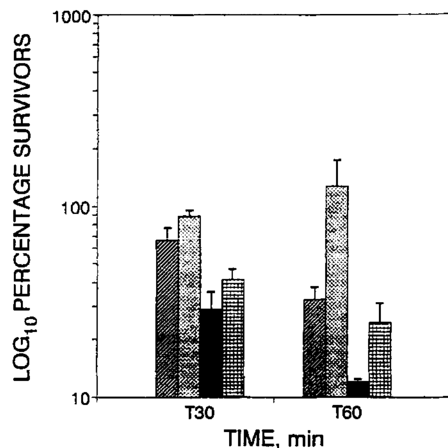

Fig. 2. The differing abilities of slow-grown (DT 60 min) and fast-grown (DT 24 min) exponential phase iron-rich (TSB + Fe) and iron-poor (TSB − Fe) phenotypes of *S. aureus* 2645 to survive in WBK. Whole blood and cocci were combined in a final ratio of ~ 1:20. Viable counts were determined at 30 min (T30) and 60 min (T60), and survival expressed as a percentage of the original challenge; mean ± S.D. of three experiments in duplicate, bars denote S.D.. These experiments were performed simultaneously with CL (Fig. 1). Columns indicate, from left to right: DT 60 min, TSB + Fe; DT 60 min, TSB − Fe; DT 24 min, TSB + Fe; DT 24 min, TSB − Fe.

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Table 2
The associations of slow-grown (DT 60 min) and fast-grown (DT 24 min) exponential phase iron-rich (TSB + Fe) and iron-poor (TSB – Fe) phenotypes of *S. aureus* with PMN in whole blood killing (WBK; see also Fig. 3)

Phenotype	Cocci associated with each of 10 PMN [a]		
DT 60 min:			
TSB + Fe	17.80 ± 7.712	$P < 0.0001$ [b],	$P < 0.0001$ [c]
TSB – Fe	6.067 ± 4.425		$P < 0.0001$ [c]
DT 24 min:			
TSB + Fe	29.533 ± 10.757	$P = 0.1641$ [b]	
TSB – Fe	26.500 ± 9.317		

[a] Mean ± S.D., 30 min sample (T30), typical subject, 3 experiments in duplicate.
[b] Comparison with TSB – Fe cocci; *t*-test.
[c] Comparisons of slow-grown TSB + Fe cocci with fast-grown TSB + Fe cocci, and of slow-grown TSB – Fe cocci with fast-grown TSB – Fe cocci; *t*-test.

and both fast-grown phenotypes showed near normal distributions with the latter associating in significantly greater numbers. Reportedly, exponential phase *S. aureus* did not show decreased association with PMN when grown in a complex medium containing desferrioxamine to restrict iron [17]. However, desferrioxamine can be ineffective against *S. aureus* [18], and as only a small relative difference was found in the intracellular iron content of PMN that had ingested either iron-restricted or iron-rich phenotypes [17], the cocci may not have encountered stringent restriction. We show, using rigorous growth conditions and iron depletion, not only that *S. aureus* iron status can influence events prior to phagocytosis, but that PMN associate less with relatively slow-grown *S. aureus* regardless of iron or opsonic status.

In *S. aureus* then, DT may, via surface characteristics, exert an influence quite distinct from those of blood or serum upon interactions with the immune system [2,14,19]. There could, for example, be a differential response by each phenotype to blood or serum components, because of differences in SH [20–22]. How changes in *S. aureus* DT or growth phase affect the structures that determine SH or the receptors for opsonins and siderophores is not characterised fully. Certainly here, the slow-growing, iron-stressed coccus, a phenotype resembling that found in vivo [3,4], was the least hydrophobic (most hydrophilic), expressed the least PrA, was the poorest inducer of the CL burst and the best survivor in WBK, a situation predicted by interfacial tension principles [2]. The trends in CL and WBK appeared to be due to a significantly reduced association with PMN. This association correlated positively with the relative hydrophobicity of the coccal surface. These findings might be relevant to the persistence of *S. aureus* in chronic infections.

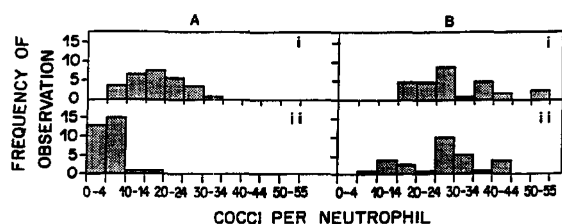

Fig. 3. The differences in distribution of the associations by slow-grown (DT 60 min) and fast-grown (DT 24 min) exponential phase iron-rich (TSB + Fe) and iron-poor (TSB – Fe) phenotypes of *S. aureus* 2645 with PMN in WBK (see also Table 2). Giemsa-stained smears were prepared from the 30 min (T30) WBK reaction mixtures (Fig. 2) and analysed by light microscope. A: DT 60 min, (i) TSB + Fe, (ii) TSB – Fe. B: DT 24 min, (i) TSB + Fe, (ii) TSB – Fe.

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References

[1] Magnusson, K.-E., Dahlgren, C., Maluszynska, G.M., Kihlstrom, E., Skogh, T., Stendahl, O., Soderlund, G. et al. (1985) Non-specific and specific recognition of bacterial and mammalian cell membranes. J. Disper. Sci. Technol. 6, 69–89.
[2] van Oss, C.J. (1978) Phagocytosis as a surface phenomenon. Annu. Rev. Microbiol. 32, 19–39.

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[3] Brown, M.R.W. and Williams, P. (1985) The influence of environment on envelope properties affecting survival of bacteria in infections. Annu. Rev. Microbiol. 39, 527–556.

[4] Dalhoff, A. (1985) Differences between bacteria grown in vitro and in vivo. J. Antimicrob. Chemother. 15, Suppl. A, 175–195.

[5] Schade, A. and Caroline, L. (1944) Raw hen egg white and the role of iron in growth inhibition of *Shigella dysenteriae*, *Staphylococcus aureus*, *Escherichia coli* and *Saccharomyces cerevisiae*. Science 100, 14–1.

[6] Domingue, P.A.G, Mottle, B., Morck, D.W., Brown, M.R.W. and Costerton, J.W. (1990) A simplified rapid method for the removal of iron and other cations from complex media. J. Microbiol. Methods 12, 13–22.

[7] Power, M.E., Olson, M.E., Domingue, P.A.G. and Costerton, J.W. (1990) A rat model of *Staphylococcus aureus* chronic osteomyelitis that provides a suitable system for studying the human infection. J. Med. Microbiol. 33, 189–198.

[8] Domingue, P.A.G., Schwarzinger, E. and Brown, M.R.W. (1989) Growth rate, iron depletion and a sub-inhibitory concentration of penicillin G affect the surface hydrophobicity of *Staphylococcus aureus*. In: The Influence of Antibiotics on the Host-Parasite Relationship (Gillissen, G., Opferkuch, W., Peters, G. and Pulverer G., Eds.), Volume 3, pp. 50–62. Springer-Verlag, Berlin.

[9] Domingue, P.A.G., Lambert, P.A. and Brown, M.R.W. (1989) Iron depletion alters surface-associated properties of *Staphylococcus aureus* and its association to human neutrophils in chemiluminescence. FEMS Microbiol. Lett. 59, 265–268.

[10] Metcalf, J.A., Gallin, J.I., Nauseef, W.M. and Root, R.K. (1986) Laboratory Manual of Neutrophil Function, Raven Press, New York.

[11] Al-Hadithy, H., Addison, I.E. and Goldstone, A.H. (1981) A rapid whole blood technique for assessment of neutrophil phagocytosis and killing. J. Clin. Lab. Haematol. 3, 85–88.

[12] Ellison III, R.T., Giehl, T.J. and LaForce, F.M. (1988) Damage of the outer membrane of enteric Gram-negative bacteria by lactoferrin and transferrin. Infect. Immun. 56, 2774–81.

[13] Jonsson, P. and Wadstrom, T. (1983) High surface hydrophobicity of *Staphylococcus aureus* as revealed by hydrophobic interaction chromatography. Curr. Microbiol. 8, 347–353.

[14] Tufano, M.A., Romano-Carratelli, C., Sommese, L., Bentivoglio, C. and Galdiero, F. (1985) Modifications of surface properties in some enteropathogenic serogroups of *Escherichia coli*. Microbiologia 8, 181–190.

[15] Hoepeleman, I.M., Bezemer, W.A., Vandenbrouke-Grauls, C.M.J.E., Marx, J.J.J.M. and Verhoef, J. (1990) Bacterial iron enhances oxygen radical-mediated killing of *Staphylococcus aureus* by phagocytes. Infect. Immun. 58, 26–31.

[16] Repine, J.E., Fox, R.B., Berger, E.M. and Harada, R.N. (1981) Effect of staphylococcal iron content on the killing of *S. aureus* by polymorphonuclear leukocytes. Infect. Immun. 32, 407–410.

[17] Cohen, M.S., Britigan, B.E., Chai, Y.S., Pou, S., Roeder, T.L. and Rosen, G.M. (1991) Phagocyte-derived free radicals stimulated by ingestion of iron-rich *Staphylococcus aureus*: A spin-trapping study. J. Infect. Dis. 163, 819–24.

[18] Brock, J.H. and Ng, J. (1983) The effect of desferrioxamine on the growth of *Staphylococcus aureus*, *Yersinia enterocolitica* and *Streptococcus faecalis* in human serum: uptake of desferrioxamine-bound iron. FEMS Microbiol. Lett. 20, 439–442

[19] Maluszynska, G.M., Stendahl, O. and Magnusson, K.-E. (1985) Interaction between human polymorphonuclear leukocytes (PMNL) and bacteria cultivated in aerobic and anaerobic conditions. Acta. Pathol. Microbiol. Immunol. Scand. B, 93B, 139–143.

[20] van Bronswijk, H., Verbrugh, H.A., Heezzius, C.J.M., Renders, N.H.M., Fleer, A., van der Meulen, J., Oe, P.L and Verhoef, J. (1989) Heterogeneity in opsonic requirements of *Staphylococcus epidermidis*: relative importance of surface hydrophobicity, capsules and slime. Immunology 67, 81–86.

[21] Liang, O.D., Asencio, F., Vazquez-Juarez, R. and Wadstrom, T. (1993) Binding of collagen, fibronectin, lactoferrin, laminin, vitrionectin and heparan sulphate to *Staphylococcus aureus* strain V8 at various growth phases and under nutrient stress conditions. Int. J. Med. Microbiol. Virol. Parasitol. Infect. Dis. 279, 180–190.

[22] Ljungh, A. and Wadstrom, T. (1995) Growth conditions influence expression of cell surface hydrophobicity of staphylococci and other wound infection pathogens. Microbiol. Immunol. 39, 753–757.

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