

RESEARCH NOTE

Open Access



The *agr* quorum sensing system in *Staphylococcus aureus* cells mediates death of sub-population

Wilhelm Paulander¹, Anders Nissen Varming^{1,2}, Martin Saxtorph Bojer¹, Cathrine Friberg^{1,3}, Kristoffer Bæk^{1,4} and Hanne Ingmer^{1*}

Abstract

Objective: In the human pathogen, *Staphylococcus aureus*, the *agr* quorum sensing system controls expression of a multitude of virulence factors and yet, *agr* negative cells frequently arise both in the laboratory and in some infections. The aim of this study was to examine the possible reasons behind this phenomenon.

Results: We examined viability of wild type and *agr* mutant cell cultures using a live-dead stain and observed that in stationary phase, 3% of the wild type population became non-viable whereas for *agr* mutant cells non-viable cells were barely detectable. The effect appears to be mediated by RNAIII, the effector molecule of *agr*, as ectopic overexpression of RNAIII resulted in 60% of the population becoming non-viable. This effect was not due to toxicity from delta toxin that is encoded by the *hld* gene located within RNAIII as *hld* overexpression did not cause cell death. Importantly, lysed *S. aureus* cells promoted bacterial growth. Our data suggest that RNAIII mediated cell death of *agr* positive but not *agr* negative cells provides a selective advantage to the *agr* negative cell population and may contribute to the common appearance of *agr* negative cells in *S. aureus* populations.

Keywords: *S. aureus*, *agr*, Quorum sensing, Cell death, Autolysis, Competition, Fitness

Introduction

Staphylococcus aureus is an opportunistic human pathogen that can cause a variety of infections [1]. The expression of virulence factors is to a large extent controlled by the *agr* quorum sensing (QS) system composed of the response regulator, AgrA and the sensor histidine kinase, AgrC that in response to auto-inducing peptides expresses a regulatory RNA, RNAIII. At high cell density RNAIII mediates the transition from production of host matrix binding and immune evasion proteins to expression of a large number of extracellular toxins including the α -hemolysin encoded by *hla* [2]. Within RNAIII itself a toxin, the δ toxin, is also encoded [3]. *S. aureus* QS has been demonstrated to be important for virulence in several animal models of acute infection, including

infective endocarditis, skin and soft tissue infections and septic arthritis [4–6]. Yet *S. aureus* QS defective mutants are commonly found in clinical isolates and they are associated with a wide range of infections such as persistent bacteremia; infections of the lungs of cystic fibrosis patients and with higher mortality in general [7–11]. Also, in the laboratory they arise spontaneously at high frequencies [12–14]. Recently we showed that exposure to sub-lethal antibiotic concentrations increases the fitness cost of the *agr* system by inducing RNAIII expression levels [15]. The fitness advantage of the *agr* mutant over the wild type (WT) strain could not be related to any differences in exponential growth rate and was only detected in competition between the two strains [15]. These observations prompted us to examine the hypothesis that the apparent fitness advantage of the *agr* mutant cells compared to the WT can be explained by differences in viability.

*Correspondence: hi@sund.ku.dk

¹ Department of Veterinary and Animal Sciences, University of Copenhagen, Frederiksberg, Denmark

Full list of author information is available at the end of the article



Main text

Methods

Bacterial strains and growth conditions

Staphylococcus aureus strains (Additional file 1: Table S1) were grown in Tryptic Soy Broth (TSB) containing 2.5 g/l glucose (Sigma-Aldrich) or in Bacto™ Tryptic Soy Broth without glucose, Benton Dickson (BD286220). Blood agar plates contained 1.5% Agar (Difco) and 5% calf's blood. Antibiotics were added in the following concentrations: tetracycline 2 µg/ml; chloramphenicol 10 µg/ml; erythromycin 10 µg/ml (Sigma-Aldrich). Mutations and plasmids were transferred by transduction using phage φ11 [16, 17]. Transposon mutant clones were obtained from the Network on Antimicrobial Resistance in *S. aureus* (NARSA). *agr* activity was assayed on blood agar plates and hemolysis was scored for approximately 100 colonies.

For *hld* overexpression, pTX_Δ-RNAIII, was equipped with an *E. coli* replication origin and ampicillin selection marker from pUC19 using primers pUC-1 and pUC-2 (Additional file 1: Table S2) through *SmaI/SacI* restriction sites yielding plasmid pTX_Δ-RNAIII-pucori. The *hld* coding sequence was amplified using primers Hld1 and Hld2 (Additional file 1: Table S2) followed by the substitution of RNAIII for *hld* using restriction sites *BamHI/MluI* yielding plasmid pTX_Δ-Hld-pucori.

Competition experiment

Five independent cultures of Δ*agrA* plus WT cells were grown in TSB with or without glucose for 100 generations (10 passages) with 1 × 10⁶ bacteria transferred daily into fresh growth medium. The ratio of the tetracycline-resistant Δ*agrA* mutant cells to WT cells was determined on TSB agar plates with and without tetracycline after 30, 50 and after 100 generations of growth.

Live/dead staining

Staining was performed with thiazol orange (TO) (Sigma-Aldrich) at 0.168 µM and propidium iodide (PI) (Sigma-Aldrich) at 8 µM, staining 10⁶ cells/ml for 15 min under dark conditions at room temperature. The flow cytometry data was recorded with a BD Biosciences Accuri C6 flow cytometer counting 50,000 cells at a flow rate of 35 µl/min and with a core size of 16 µm. Stained cells were excited with a 488 nm argon laser and emission was detected with the FL1 emission filter at 533/30 nm using FL1 photomultiplier tub and in FL-3 emission filter at 670 nm using FL3 photomultiplier tub.

Quantitation of RNAIII expression and eDNA levels by real-time qPCR

The SV RNeasy Mini Kit (Qiagen) was used for isolation of RNA; cDNA RT kit (Applied Biosystems) for cDNA

synthesis (using an RNase inhibitor, Applied Biosystems) and the FastStart Essential DNA Green Master (Roche) for qPCR in a Lightcycler 96 (Roche) with the primers listed in Additional file 1: Table S2. Data analysis was performed in the LightCycler Application Software, version 1.1 (Roche). Extracellular, chromosomal DNA (eDNA) was quantified by qPCR amplifying the *ileS* sequence directly on 100-fold diluted and heat-treated culture supernatants. The eDNA concentration in the supernatants were subsequently calculated from a standard curve of purified genomic DNA and normalized using an exogenous DNA spike which was added to all samples. The DNA spike was PCR product of the GFP gene and 10,000 copies were added to each qPCR reaction (primers used are listed in Additional file 1: Table S2).

Growth potential of lysed cells

Staphylococcus aureus cells in TSB were lysed by applying bead-beating (FastPrep®-24, MP Biomedicals). Increasing concentrations of lysate were added to TSB diluted 1:10 with water, inoculated with *S. aureus* Newman overnight cultures (diluted 1:100) and incubated in a Bioscreen C reader (Thermo Labsystems) at 37 °C for 24 h. Technical quadruplicates and biological triplicates were included for each condition.

Results

Cell death is reduced in *agrA* and RNAIII mutant cells

Initially we assessed the frequency with which *agr* negative cells arise in strain Newman by passaging five individual WT colonies in serial batch cultures and determining the frequency of hemolysin negative mutants on blood agar plates. Although only a fraction of *agr* mutations will eliminate hemolysis it has previously been used as an indicator of *agr* activity [14]. Non-hemolytic colonies appeared on day 7 and by day 19 haemolytic colonies could only be detected in one lineage (Additional file 1: Figure S1). Thus, in line with findings for other *S. aureus* strains [14], *agr* mutants readily arise in cultures of stain Newman during serial passage.

Since the selection for *agr* negative cells during the serial passage cannot readily be explained by differences in growth rate and is primarily detected in competition with WT cells [14, 15] we examined the viability of *agr* positive and negative cells. Stationary phase cultures of Newman WT, Δ*agrA* and ΔRNAIII mutant derivatives were live/dead stained with propidium iodide (PI) and thiazole orange (TO) and analysed by flow cytometry. Interestingly, we observed a significantly higher fraction ($p < 0.05$) of dead WT cells compared to Δ*agrA* or ΔRNAIII mutant cells (Fig. 1a) indicating that a fraction of *agr* positive cells lose viability by a process not taking place in Δ*agrA* or ΔRNAIII mutant cells.

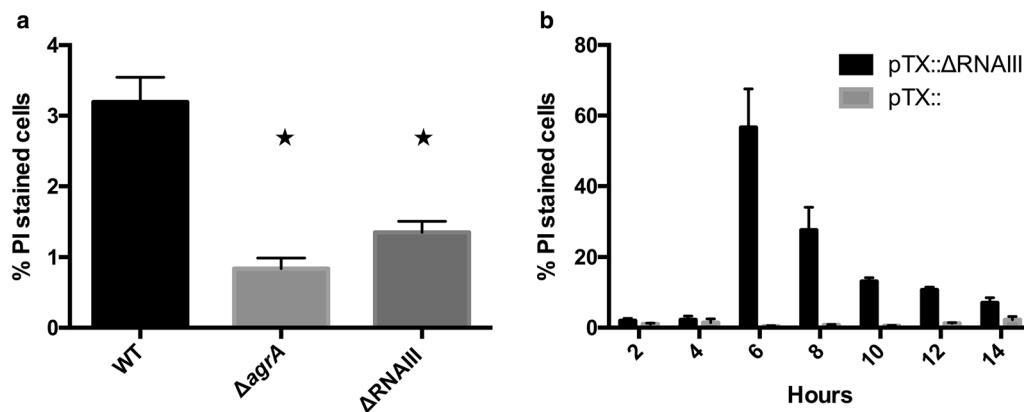


Fig. 1 *agr* and RNAIII influence cell viability. **a** Cultures of WT, $\Delta agrA$ and $\Delta RNAIII$ mutant were stained with PI and TO and the percentage of dead cells determined by flowcytometry (An asterisk indicates a *p*-value of < 0.05 when compared to WT by Student's *T*-test). **b** The percentage of dead cells was determined by staining and flowcytometry at various time points after inoculation of WT cells carrying either vector (pTX Δ) or the RNA overproducing plasmid (pTX Δ : $\Delta RNAIII$). Bars represent the mean and the standard deviation from biological triplicates

RNAIII overexpression induces lysis

To address if RNAIII may be the factor reducing the viability in *agr* positive cells, we examined populations of WT cells containing an empty vector, pTX Δ , or a plasmid constitutively expressing RNAIII, pTX Δ :RNAIII. Cultures of both strains were inoculated to a cell density of 5×10^6 colony forming units per ml, growth as well as the live/dead ratio was continuously monitored with flow cytometry. We observed that with the pTX Δ :RNAIII construct, substantial cell death occurred after 6 h of growth with more than 60% of the population stained as dead cells (Fig. 1b) whereas few dead cells were observed in cells carrying the empty vector, pTX Δ . At this timepoint the cells carrying pTX Δ :RNAIII overproduced RNAIII tenfold above the level in cells carrying the vector (Additional file 1: Figure S2). Upon progression into stationary growth phase, the number of cells stained as dead decreased with RNAIII overproduction (Fig. 1b) indicating that dead cells lyse and consequently are not detected in the flow cytometer. This observation was confirmed by monitoring the release of chromosomal DNA by qPCR. After 7 h of growth, extracellular DNA (eDNA) could only be observed in the supernatant of cells with RNAIII overproduction and it appeared at a concentration of $13.9 \mu\text{g/ml}$ (± 4.0) eDNA detected, corresponding to the DNA content of 5×10^9 *S. aureus* cells/ml. To assess whether the pronounced cell death observed with RNAIII overexpression was due to overproduction of the δ -toxin encoded by *hld* within the RNAIII transcript, we overproduced δ -toxin from a construct not expressing RNAIII. To this end we removed the entire RNAIII-encoding region and inserted *hld* with the same Shine Dalgarno sequence as on the native transcript. With this plasmid cell death was reduced to only 10% of that seen

with the RNAIII-overproducing plasmid showing that the effect is mediated via RNAIII and not the δ -toxin.

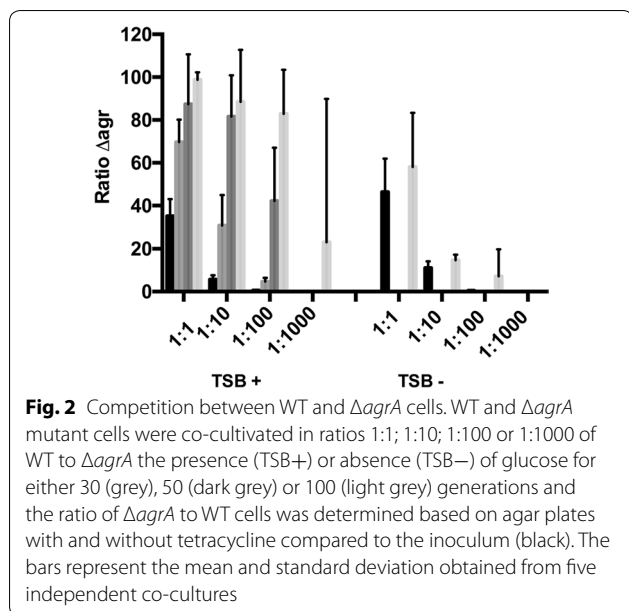
Bacterial lysis releases resources supporting growth

Since dead cells potentially are cannibalized we examined whether lysed *S. aureus* cells could support growth. For this purpose, we inoculated WT cells in dilute TSB broth ($0.1 \times$ TSB) supplemented with varying amounts of staphylococcal lysate obtained from mechanical disruption of *S. aureus* WT cells and observed that increasing amounts of lysate stimulates growth as observed by a higher final optical cell density (Additional file 1: Figure S3).

Staphylococcus aureus is known to encode a number of autolysins and we speculated that one of these might be responsible for the cell death. However, upon transduction of mutations in *lrgB*, *cidA*, *lrgA*, *lytM*, *tagX*, a lysM domain protein (NE1640) and an autolysin (NE1948) from the NARSA transposon library into Newman + pTX Δ :RNAIII none of the mutations altered the lysis phenotype elicited by RNAIII overproduction (data not shown) indicating that the examined gene products are not responsible for the RNAIII mediated cell death.

Modulation of RNAIII expression eliminates the competitive advantage of *agr* negative cells

To determine if RNAIII expression levels influences the competition between WT and *agr* mutant cells we competed $\Delta agrA$ with WT cells and observed that $\Delta agrA$ cells quickly outcompeted the WT in regular TSB medium (Fig. 2) while this was not the case in TSB lacking glucose where RNAIII expression has been demonstrated to be reduced [18] (Fig. 2). These data suggest that



the competitive advantage of being *agr* negative is associated with less RNAIII expression and less lysis of cells.

Discussion

Here we show that a small fraction of a WT *S. aureus* population undergoes cell death and that this does not take place in mutant cells lacking the *agr* QS system. Since both *agr* positive and negative cells multiply on lysed staphylococcal cells, *agr* negative cells have an advantage over WT cells. We propose that this phenomenon contributes to the frequent manifestation of *agr* mutant cells both in vivo and in vitro during serial passage [14]. Our results agree with previous findings that the apparent fitness advantage of *agr* negative cells is particularly evident in competition assays [15]. Currently we do not know the molecular details of the killing process nor the mechanism behind the stochasticity by which it occurs. However, it has been noted that *agr* mutant cells are less prone to Triton X-100 mediated lysis and are more resistant to lysis by Penicillin G compared to wild type cells [19] indicating that there is an overall basic physiological difference between *agr* positive and negative cells.

In *P. aeruginosa* QS has also been linked with decreased viability as a mutant lacking the *las* QS system resists autolysis at high cell densities resulting in about tenfold increase in *lasR* mutant-to-wild-type ratio in mixed cultures [20]. Interestingly QS negative cells of both *S. aureus* and *P. aeruginosa* appear under chronic infections experiencing prolonged antibiotic exposure [20]. In *S. aureus*, some antibiotics are known to increase expression of RNAIII [9, 21]. We speculate

that this induction may lead to increased cell death in WT populations of cells and enhanced the appearance of *agr* mutant cells.

The biological impact of differential death of *agr* positive cells remain obscure. It may contribute to biofilm formation via the DNA released [22] but it may also serve the purpose of establishing mixed populations of *agr* positive and negative cells. As the fraction of *agr* negative cells increases, the induction of *agr* expression in WT cells will decrease and consequently also the RNAIII mediated lethality. In *Salmonella enterica* serovar Typhimurium it has been shown that expression of a phenotypically avirulent subpopulation promotes the evolutionary stability of virulence (35) and similar cooperation may take place in *S. aureus*.

Limitations

While the phenomenon reported in our study is observed for several strains of *S. aureus* we do not know the extent to which cell death in stationary phase occurs in clinical strains.

Additional file

Additional file 1: Figure S1. Hemolysis negative cells arise in *S. aureus* Newman. Five parallel cultures of WT were passaged in TSB for 24 days. Every second day suitable dilutions were plated out on TSB agar with 5 % calf's blood and each colony was scored for hemolysis by comparing to WT freshly inoculated from the freeze stock. Zones of hemolysis smaller than ~0,5 mm were scored as hemolysis negative. **Figure S2.** RNAIII overexpression with pTX Δ RNAIII. RT-qPCR was used to measure expression of RNAIII in *S. aureus* Newman carrying vector (pTX Δ) or RNAIII overproducing plasmid (pTX Δ RNAIII) after 6 hours of growth in TSB. Data represent three biological replicates and are shown as mean ratios normalized to a run calibrator of genomic DNA. Error bars represent the standard deviation. **Figure S3.** Lysed bacteria supports growth. WT cells were grown in diluted 0.1xTSB supplemented with increasing amounts of bacterial lysate, and growth was measured in a Bioscreen at OD₆₀₀. The experiment was performed with biological triplicates for each condition and the data represent the mean OD₆₀₀ and standard deviation. **Table S1.** Strains and plasmids used in this study. **Table S2.** Oligonucleotides used in this study.

Abbreviations

QS: quorum sensing; TSB: tryptic soy broth; eDNA: extracellular chromosomal DNA.

Authors' contributions

WP, ANV and HI developed the study design, WP, ANV, MSB, CF, KB conducted experiments, WP, ANV, HI analysed the data; ANV, WP and HI wrote the manuscript. All authors read and approved the manuscript.

Author details

¹ Department of Veterinary and Animal Sciences, University of Copenhagen, Frederiksberg, Denmark. ² Present Address: ALK-Abelló, Horsholm, Denmark. ³ Present Address: Novo Nordisk, Gentofte, Denmark. ⁴ Imperial College London, London, UK.

Acknowledgements

We acknowledge the NARSA program for providing isolates used in this study.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article (and its additional file(s)).

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

Funding

HI was supported by grants from the Danish Research Council of Independent Research (12-127417) and Danish National Research Foundation's Centre of Excellence Bacterial Stress Response and Persistence (grant identifier DNRF120). WP was supported by grants from the Danish Research Council of Independent Research (09-069656) and Ung Eliteforskerpris (09-076146).

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 30 May 2018 Accepted: 12 July 2018

Published online: 24 July 2018

References

- Lowy FD. *Staphylococcus aureus* infections. *N Engl J Med*. 1998;339:520–32.
- 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;12:3967–75.
- Janzon L, Löfdahl S, Arvidson S. Identification and nucleotide sequence of the delta-lysin gene, *hld*, adjacent to the accessory gene regulator (*agr*) of *Staphylococcus aureus*. *Mol Gen Genet*. 1989;219:480–5.
- Cheung AL, Eberhardt KJ, Chung E, Yeaman MR, Sullam PM, Ramos M, Bayer AS. Diminished virulence of a *sar-agr* mutant of *Staphylococcus aureus* in the rabbit model of endocarditis. *J Clin Invest*. 1994;94:1815–22.
- Kobayashi SD, Malachowa N, Whitney AR, Braughton KR, Gardner DJ, Long D, Bubeck-Wardenburg J, Schneewind O, Otto M, DeLeo FR. Comparative analysis of USA300 virulence determinants in a rabbit model of skin and soft tissue infection. *J Infect Dis*. 2011;204:937–41.
- Abdelnour A, Arvidson S, Bremell T, Rydén C, Tarkowski A. The accessory gene regulator (*agr*) controls *Staphylococcus aureus* virulence in a murine arthritis model. *Infect Immun*. 1993;61:3879–85.
- Fowler VG Jr, Sakoulas G, McIntyre LM, Meka VG, Arbeit RD, Cabell CH, Stryjewski ME, Eliopoulos GM, Reller LB, Corey GR, Jones T, Lucindo N, Yeaman MR, Bayer AS. Persistent bacteremia due to methicillin-resistant *Staphylococcus aureus* infection is associated with *agr* dysfunction and low-level in vitro resistance to thrombin-induced platelet microbicidal protein. *J Infect Dis*. 2004;190:1140–9.
- Goerke C, Campana S, Bayer MG, Döring G, Botzenhart K, Wolz C. Direct quantitative transcript analysis of the *agr* regulon of *Staphylococcus aureus* during human infection in comparison to the expression profile in vitro. *Infect Immun*. 2006;68:1304–11.
- Traber KE, Lee E, Benson S, Corrigan R, Cantera M, Shospin B, Novick RP. *agr* function in clinical *Staphylococcus aureus* isolates. *Microbiology*. 2008;154:2265–74.
- Schweizer ML, Furuno JP, Sakoulas G, Johnson JK, Harris AD, Shardell MD, McGregor JC, Thom KA, Perencevich EN. Increased mortality with accessory gene regulator (*agr*) dysfunction in *Staphylococcus aureus* among bacteremic patients. *Antimicrob Agents Chemother*. 2011;55:1082–7.
- Painter KL, Krishna A, Wigneshweraraj S, Edwards AM. What role does the quorum-sensing accessory gene regulator system play during *Staphylococcus aureus* bacteremia? *Trends Microbiol*. 2014;22:676–85.
- Chen J, Novick RP. *svrA*, a multi-drug exporter, does not control *agr*. *Microbiology*. 2007;153:1604–8.
- Adhikari RP, Arvidson S, Novick RP. A nonsense mutation in *agrA* accounts for the defect in *agr* expression and the avirulence of *Staphylococcus aureus* 8325-4 *traP:kan*. *Infect Immun*. 2007;75:4534–40.
- Somerville GA, Beres SB, Fitzgerald JR, DeLeo FR, Cole RL, Hoff JS, Musser JM. In vitro serial passage of *Staphylococcus aureus*: changes in physiology, virulence factor production, and *agr* nucleotide sequence. *J Bacteriol*. 2002;184:1430–7.
- Paulander W, Nissen Varming A, Baek KT, Haaber J, Frees D, Ingmer H. Antibiotic-mediated selection of quorum-sensing-negative *Staphylococcus aureus*. *mBio*. 2013;3:e00459-12.
- Novick RP. Genetic systems in staphylococci. *Methods in enzymology*, vol. 204. Cambridge: Academic Press; 1991. p. 587–636.
- Schenk S, Laddaga RA. Improved method for electroporation of *Staphylococcus aureus*. *FEMS Microbiol Lett*. 1992;73:133–8.
- Seidl K, Stucki M, Ruegg M, Goerke C, Wolz C, Harris L, Berger-Bächi B, Bischoff M. *Staphylococcus aureus* CcpA affects virulence determinant production and antibiotic resistance. *Antimicrob Agents Chemother*. 2006;50:1183–94.
- Fujimoto DF, Bayles KW. Opposing roles of the *Staphylococcus aureus* virulence regulators, Agr and Sar, in Triton X-100- and penicillin-induced autolysis. *J Bacteriol*. 1998;180:3724–6.
- Heurlier K, Denervaud V, Haenni M, Guy L, Krishnapillai V, Haas D. Quorum-sensing-negative (*lasR*) mutants of *Pseudomonas aeruginosa* avoid cell lysis and death. *J Bacteriol*. 2005;187:4875–83.
- Joo HS, Chan JL, Cheung GY, Otto M. Subinhibitory concentrations of protein synthesis-inhibiting antibiotics promote increased expression of the *agr* virulence regulator and production of phenol-soluble modulins in community-associated methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother*. 2010;54:4942–4.
- Grande R, Nistico L, Sambanthamoorthy K, Longwell M, Iannitelli A, Cellini L, Di Stefano A, Hall Stoodley L, Stoodley P. Temporal expression of *agrB*, *cidA*, and *alsS* in the early development of *Staphylococcus aureus* UAMS-1 biofilm formation and the structural role of extracellular DNA and carbohydrates. *Pathog Dis*. 2014;70:414–22.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions

