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Gene expression analysis of monospecies *Salmonella* Typhimurium biofilms using Differential Fluorescence Induction

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ABSTRACT

Bacterial biofilm formation is an important cause of environmental persistence of food-borne pathogens, such as *Salmonella* Typhimurium. As the ensemble of bacterial cells within a biofilm represents different physiological states, even for monospecies biofilms, gene expression patterns in these multicellular assemblages show a high degree of heterogeneity. This heterogeneity might mask differential gene expression that occurs only in subpopulations of the entire biofilm population when using methods that average expression output. In an attempt to address this problem and to refine expression analysis in biofilm studies, we used the Differential Fluorescence Induction (DFI) technique to gain more insight in *S.* Typhimurium biofilm gene expression. Using this single cell approach, we were able to identify 26 genetic loci showing biofilm specific increased expression. For a selected number of identified genes, we confirmed the DFI results by the construction of defined promoter fusions, measurement of relative gene expression levels and construction of mutants. Overall, we have shown for the first time that the DFI technique can be used in biofilm research. The fact that this analysis revealed genes that have not been linked with *Salmonella* biofilm formation in previous studies using different approaches illustrates that no single technique, *in casu* biofilm formation, is able to identify all genes related to a given phenotype.

1. Introduction

During the last decades, it has become increasingly clear that bacteria, including pathogens such as *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), grow predominantly as biofilms in most of their natural habitats, rather than in planktonic mode (Hall-Stoodley et al., 2004). *S.* Typhimurium is a Gram-negative, enteropathogenic bacterium that causes host-specific diseases ranging from self-limiting food-borne gastroenteritis to life-threatening systemic infections. *Salmonella* is capable of forming microcolonies and even mature biofilms on a wide diversity of surfaces, ranging from abiotic (Kusumaningrum et al., 2003; Latasa et al., 2005; Romling et al., 1998) to biotic (Barak et al., 2008; Boddicker et al., 2002; Brandl and Mandrell, 2002; Prouty et al., 2002) ones. Bacterial biofilms can be defined as structured communities of bacterial cells enclosed in a self-produced matrix, adhering to inert or living surfaces (Costerton et al., 1999). Biofilm formation has been stated as a potential cause of the emerging (multi)drug resistance (Lewis, 2008), because of the protecting action of the self-produced matrix (Fux et al., 2005) and adaptation mechanisms of the bacteria residing in these multicellular structures. Furthermore, *Salmonella* biofilm formation is an important survival strategy in non-host environments, which are fundamentally different from typical host environments (Mouslim et al., 2002; Romling et al., 2007), a strategy to induce chronic infections (Costerton et al., 1995; Davey and O'Toole, 2000) and even a possible way to colonize host organisms (Boddicker, et al., 2002; Prouty and Gunn, 2003). Taken together, *Salmonella* biofilm formation can be seen as an essential and integral part of the pathogen's life cycle and a source of reappearing infections by this pathogen (Rasschaert et al., 2007).

Bacterial cells residing in biofilms are not only physiologically distinct from planktonic cells (with different gene expression patterns), but also vary from each other spatially, temporally and genetically as the biofilm formation proceeds (Sauer et al., 2002; Stewart and Franklin, 2008; Stoodley et al., 2002). High-throughput DNA microarray studies have been conducted to study biofilm formation in many model organisms and have identified a large number of genes showing differential expression under biofilm conditions (*e.g.* (Beenken et al., 2004; Beloin et al., 2004; Hamilton et al., 2009; Ren et al., 2004; Schembri et al., 2003; Shemesh et al., 2008; Whiteley et al., 2001)). This transcriptional profiling technique, however, generates a global value for the whole biofilm population (An and Parsek, 2007; Lazazzera, 2005; Stewart and Franklin, 2008) and as such, differences in gene expression patterns of subpopulations within biofilms are not taken into account.

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In an attempt to address this problem and to refine expression analysis in biofilm studies, we decided to use the Differential Fluorescence Induction (DFI) single cell approach to study *Salmonella* biofilm formation. This technique was introduced by the Falkow group to sort *S.* Typhimurium clones differentially expressing GFP in low pH environments and within host cells (Valdivia and Falkow, 1996, 1997). DFI basically is an enrichment strategy using fragments of bacterial genomic DNA, cloned upstream of a promoterless *gfp*, and a flow cytometer with sorting module to monitor promoter activity. In this study, the genetic enrichment was performed by alternating rounds of positive (biofilm-inducing) and negative (planktonic conditions) selection of the bacterial population. This resulted progressively in the generation of bacterial subpopulations showing enrichment in biofilm upregulated inserts. Subsequent sequence determination of the genomic inserts in these enriched pools led to identification of DNA sequences that caused increased expression of the promoterless *gfp* gene in biofilm conditions in non-host environments. Using this enrichment technique in a biofilm context, we identified 26 genetic loci showing *Salmonella* biofilm specific induction of which 17 coincided with promoter regions of already annotated genes. For a selected number of DFI identified genes, we confirmed the results of the screening by the construction of defined promoter fusions. We also measured relative gene expression levels with qRT-PCR for a selection of DFI-retrieved genes to compare differential expression directly at the RNA level. Finally, for some of the identified genes, we investigated their impact on *Salmonella* biofilm formation by constructing and analyzing corresponding mutants.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

All strains and plasmids used in this study are listed in Table 1. In the planktonic state, *S.* Typhimurium strains were grown with aeration at 37 °C in Luria-Bertani (LB) broth (Sambrook and Russel, 2001) or on LB plates containing 15 g/l agar (Invitrogen). If appropriate, antibiotics were added at the following concentrations: ampicillin (Ap), 100 μg/ml; chloramphenicol (Cm), 25 μg/ml and streptomycin (Sm), 25 μg/ml. Tryptic soy broth (BD Biosciences, 30 g/l) diluted 1/20 (TSB 1/20) was used for biofilm formation assays (De Keersmaecker et al., 2005).

Standard protocols were used for molecular cloning (Sambrook and Russel, 2001). Restriction enzymes were purchased from New England Biolabs and used according to the manufacturer's instructions. Cloning steps were performed using *E. coli* DH5α and *E. coli* TOP10F′ (Sambrook and Russel, 2001) and the final, constructed plasmids were electroporated to the *S.* Typhimurium SL1344 strains using a Bio-Rad gene pulser. All primers used, as well as their purposes, are listed in Table 2. The sequences used for primer construction were obtained from the complete genome sequence of *S.* Typhimurium SL1344 (Hoiseth and Stocker, 1981), as available via the website of the Sanger Institute (U.K.), (http://www.sanger.ac.uk/Projects/Salmonella). Reporter plasmids pCMPG5521, pCMPG5532, pCMPG5533 and pCMPG5539 were constructed by cloning the PCR-amplified *csgD*, *potF*, STM1851 and *csgB* promoter regions, respectively, as a BamHI (for pCMPG5521 and pCMPG5539) or XbaI/BamHI (for pCMPG5532 and pCMPG5533) fragment into pFPV25. Complementation plasmids pCMPG5522, pCMPG5531 and pCMPG5538 were constructed by cloning the PCR-amplified *potF*, *potFGHI* and *sitABCD* coding sequences, as EcoRI (pCMPG5522) and XbaI/BamHI (pCMPG5531, pCMPG5538) fragments downstream of the constitutive *nptII* promoter into the RK2 based plasmid pFAJ1708 (Dombrecht et al., 2001). As such, complementation experiments were performed using this *nptII* promoter to drive expression of the mentioned genes. Correct orientation of all fragments was checked by PCR and restriction analysis. *S.* Typhimurium SL1344 mutants were constructed using the one-step chromosomal inactivation protocol, as previously described by Datsenko and Wanner (Datsenko and

Table 1
Bacterial strains and plasmids.

Name	Description	Reference
Strains		
E. coli DH5α	F$^-$ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG* ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169*, *hsdR17*(r_K^- m_K^+), λ^-	Gibco BRL
E. coli TOP10F′	F′ {*lacI*q Tn*10*(TetR)} *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)7697 *galU galK rpsL* (StrR) *endA1 nupG*	Invitrogen
S. Typhimurium SL1344	Parent strain, *xyl hisG rpsL*; virulent; Sm^R	(Hoiseth and Stocker, 1981)
CMPG 5521	*S.* Typhimurium SL1344, *potF*::Cm	This work
CMPG 5522	*S.* Typhimurium SL1344, Δ*potF*	This work
CMPG 5537	*S.* Typhimurium SL1344, ΔSTM1851	This work
CMPG5579	*S.* Typhimurium SL1344, Δ*csgD*	This work
CMPG5584	*S.* Typhimurium SL1344, *sitA*::Cm	This work
CMPG5589	*S.* Typhimurium SL1344, Δ*cpxP*	This work
CMPG10301	*S.* Typhimurium SL1344, Δ*sanA*	This work
CMPG10305	*S.* Typhimurium SL1344, *fhuA*::Cm	This work
CMPG10309	*S.* Typhimurium SL1344, Δ*csgB*	This work
Plasmids		
pCMPG5521	*csgD* promoter cloned BamHI in pFPV25	This work
pCMPG5522	*potF* cloned EcoRI in pFAJ1708	This work
pCMPG5531	*potF* cloned XbaI/BamHI in pFAJ1708	This work
pCMPG5532	*potF* promoter cloned XbaI/BamHI in pFPV25	This work
pCMPG5533	STM1851 promoter cloned XbaI/BamHI in pFPV25	This work
pCMPG5538	*sitABCD* cloned XbaI/BamHI in pFAJ1708	This work
pCMPG5539	*csgB* promoter cloned BamHI in pFPV25	This work
pCP20	*flp*, ts-rep-[*cI857*](λ) ts, Ap^R, Cm^R	(Datsenko and Wanner, 2000)
pFPV25	Promoter-trap vector constructed by inserting an EcoRI-HindIII fragment containing a promoterless *gfpmut3* (Cormack, et al., 1996) into plasmid pED350 (colE1, *bla*, *mob*) (Derbyshire and Willetts, 1987); Ap^R	(Valdivia and Falkow, 1996)
pFPV25.1	0.6 kb *Sau*3AI fragment inserted in the BamHI site of pFPV25, containing the promoter region of *S.* Typhimurium *rpsM* encoding for the ribosomal protein S13 (constitutive promoter); Ap^R	(Valdivia and Falkow, 1996)
pKD3	Plasmid used as template for construction of *Salmonella* mutants; Ap^R, Cm^R	(Datsenko and Wanner, 2000)
pKD46	Lambda Red helper plasmid, Ap^R	(Datsenko and Wanner, 2000)
pFAJ1708	Derivative of RK-2; Ap^R; Tc^R; contains *nptII* promoter of pUC18-2	(Dombrecht et al., 2001)