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Production of Siderophores by *Serratia marcescens* and the Role of MacAB Efflux Pump in Siderophores Secretion

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Abstract Human opportunistic pathogen *Serratia marcescens* secrete siderophores to enable growth under iron-limiting conditions. Iron acquisition genes are among a few known virulence factors of *S. marcescens*. Siderophore export systems in Gram-negative bacteria are not fully understood. There is some evidence for involvement of efflux pumps in the export of synthesized enterobactin-like molecules. The goal of this study was to characterize siderophore production by two different strains of *S. marcescens*, SM6 and SR41-8000, and to evaluate the role of efflux pump MacAB in siderophore secretion by these strains. We showed that both strains produced siderophores in CAS agar assay. We further showed that both strains were able to secrete catecholate siderophores in response to iron starvation. MacAB efflux pump played a role in siderophore secretion of *S. marcescens* SR41-8000 strain but was dispensable for accumulation of these molecules in the culture supernatant of *S. marcescens* strain SM6.

Keywords *Serratia marcescens* · Siderophores · 2,3-Dihydroxybenzoic acid · Efflux pump · MacAB

1 Introduction

Serratia marcescens is a member of the *Enterobacteriaceae* family. This bacterium can cause infections of numerous organ systems and represents a growing public health problem. Virulence factors of *S. marcescens* include secretion of hydrolytic enzymes [1], lipopolysaccharide biosynthesis, hemolysin production, and iron uptake [2]. Iron is an essential element for many biological processes. To overcome iron deficiency, many bacteria synthesize siderophores, low molecular weight iron-chelating compounds [3]. Related bacterium *E. coli* secretes catecholate siderophore enterobactin. The systems involved in synthesis and uptake of enterobactin are well characterized [4]. In contrast, enterobactin export system is not fully understood. In addition to previously identified ABC-type transpoter EntS [5], several RND efflux pump, AcrB, AcrD, and MdtABC recently have been shown to be involved in export of enterobactin [6]. Our understanding of production and secretion of siderophores by *S. marcescens* is very limited. Thus, the aim of this study was to characterize siderophore production by two wild-type strains of *S. marcescens*, SM6 and SR41-8000, and to investigate the role of ABC-type efflux pump MacAB in secretion of siderophores by these strains.

2 Material and Methods

S. marcescens strains SM6 and SR41-8000 were obtained from Michael Benedik (Texas A&M University). Isogenic Δ*macAB* strains were generated using modified lambda red recombination method [7] (will be described elsewhere). Bacterial strains were initially screened for siderophore production using the CAS assay method [8]. The Arnow assay was used to detect catecholate-type siderophores [9]. 2,3-Dihydroxybenzoic acid (2.3-DHBA) (Acros) was used as a standard for calibration curve. The Atkin assay was used to test for the presence of hydroxamate-type siderophores [10].

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Desferrioxamine B (Sigma) was used as a standard for calibration curve.

Siderophore detection in solution was done using CAS liquid assay [11]. Bacteria were cultivated in MM9 (with 100 mM PIPES, 3 % casamino acids and 20 % glucose). Detection of siderophores in supernatants was performed by using CAS assay solution (2 mM aqueous CAS, 1 mM $FeCl_3$ in 10 mM HCl, 10 mM HDTMA, 4.307 g anhydrous piperazine). Equal volumes of supernatant and CAS assay solution were mixed and incubated for 5 min, followed by addition of 1/20th of volume 0.2 M 5-sulfosalicylic acid. Absorbance of the solution was measured at 630 nm. Results were presented in siderophore units calculated as described in [11].

S. *marcescens* strains were grown at 30 °C with shaking (200 rpm) for 12 h in 5 mL of LB medium containing (per L) 10 g tryptone, 5 g yeast extract 5 g NaCl. Bacterial cells were harvested by centrifugation, washed with 16 mM phosphate buffer (pH 7.0), and resuspended in 15 ml M9 minimal medium. The M9 medium contained (per L) 6.77 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl, and 20–100 mM glucose or 2–10 % glycerine, pH 6.9–7.0. Stock solutions of $MgSO_4 \times 7H_2O$ and $CaCl_2$ were added to final concentration 246 and 100 μM, respectively. M9 medium was supplemented with BIP (2.2-bipiridyl) (Sigma) to final concentration 50 μM. Control experiments were performed in the presence of 100 μM of $FeCl_3$. Bacterial growth was monitored at 600 nm (iMark™ microplate absorbance reader, BioRad, USA).

Each experiment was performed on at least three separate occasions. Statistical significance was determined by using Student's *t* test.

3 Results and Discussion

The results of the CAS assay showed that both *S. marcescens* wild-type strains, SM6 and SR41-8000, as well as their isogenic Δ*macAB* were able to produce siderophores. After 12 h of growth of *S. marcescens* strains on CAS agar plate, the medium color has changed from blue to yellow. Production of siderophores depended on the strain background. *S. marcescens* strain SR41-8000 produced more siderophores on CAS plates compared to strain SM6 (Fig. 1a).

CAS agar plate assay is a universal method for siderophore detection, but it cannot provide any information about chemical properties of iron-chelating compounds. To characterize synthesized molecules in greater details, we determined concentration of catecholate and hydroxamate siderophores in the culture supernatants using methods of Arnow and Atkin, respectively. The analysis of siderophore accumulation in the culture supernatant over 30 h of growth under iron-limiting conditions showed that initial production of catecholate siderophores by

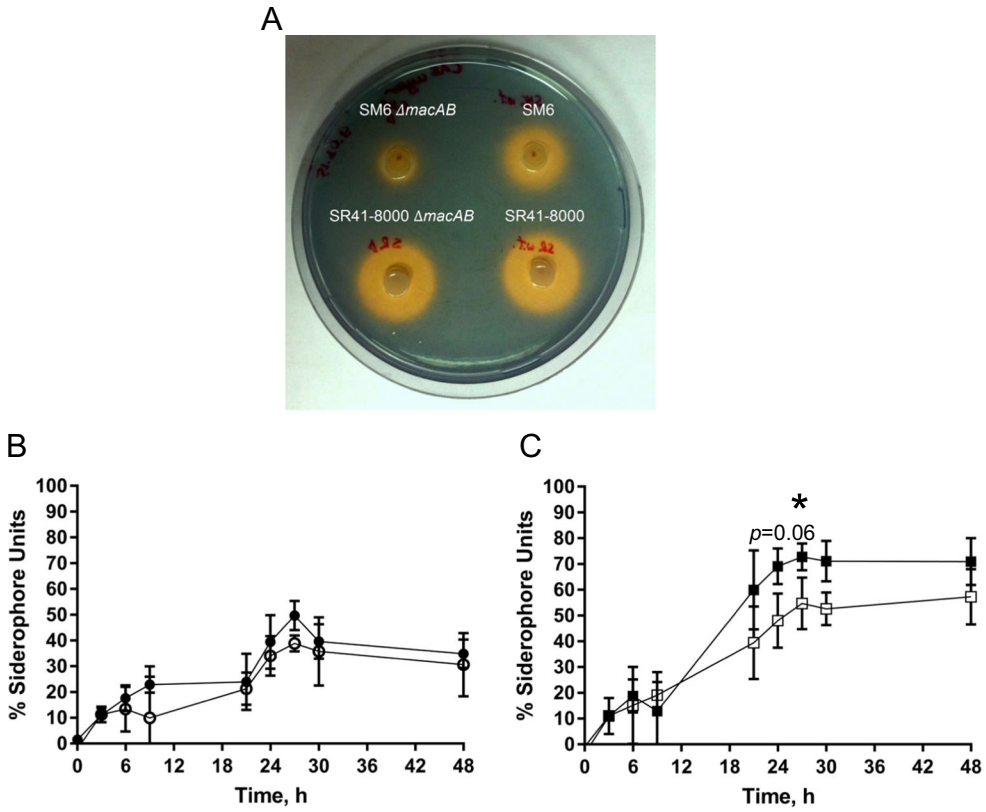

Fig. 1 Production of siderophores by *Serratia marcescens* strains SM6, SR41-8000 and their isogenic Δ*macAB* mutants. **a** All *S. marcescens* strains produce siderophores on CAS agar. **b**, **c** Secretion of siderophores by *S. marcescens* strains was evaluated in liquid CAS assay. **b** SM6—*closed circles*; SM6 Δ*macAB*—*open circles*. **c** SR41-8000—*closed squares*; SR41-8000 Δ*macAB*—*open squares*. *Asterisk* indicates $p < 0.05$ in two-tailed unpaired Student's *t* test

Table 1 Production of siderophores by *Serratia marcescens* strains SM6 and SR41-8000 after 30 h under iron-limiting conditions

Growth temperature	Carbon source	Siderophores concentration, μM	
		SM6	SR41-8000
30 °C	20 mM glucose	78.0 ± 5.69	10.0 ± 4.0
	2 % glycerin	127.0 ± 7.1	11.0 ± 3.0
37 °C	20 mM glucose	ND	ND
	2 % glycerin	ND	ND

ND not detected

S. marcescens SM6 coincided with transition to the early exponential growth phase. Siderophore concentration gradually increased over 30 h of growth and remained constant after that. It was found that low concentrations of glucose (20 mM) and glycerine (2 %) stimulated a siderophore secretion by *S. marcescens* SM6 strain at 30 °C (Table 1). Production of catecholate siderophores by *S. marcescens* SR41-8000 strain under used conditions was low, regardless of the temperature and carbon source (Table 1). Growth of bacteria in the presence of 100 μM $FeSO_4$ did not result in siderophore secretion. Production of hydroxamate-type of siderophores was not detected in neither of the *S. marcescens* strains.

To evaluate the impact of MacAB efflux pump on the secretion of siderophores, we used liquid CAS assay [11]. In agreement with CAS plate assay *S. marcescens* SM6 and its isogenic Δ*macAB* mutant strain produced significantly less siderophores compared to strain SR41-8000 (Fig. 1b, c). The role of MacAB efflux pump in secretion of siderophores by *S. marcescens* was strain-specific. Deletion of MacAB efflux pump did not play a role in accumulation of siderophores in the culture supernatant of *S. marcescens* SM6 over 48 h of growth (Fig. 1b). In contrast, secretion of siderophores by Δ*macAB* mutant strain was significantly reduced compared to its parent, *S. marcescens* SR41-8000 strain (Fig. 1c). This finding illustrates remarkable intraspecies diversity previously observed for *Serratia marcescens* [12].

4 Conclusions

Thus, both *Serratia marcescens* strains, SM6 and SR41-8000, produced catecholate siderophores under iron-limiting growth conditions. Efflux pump MacAB was involved in the secretion of these iron-chelating molecules in the strain SR41-8000 but not in the strain SM6.

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