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Disruption of the copper efflux pump (CopA) of *Serratia marcescens* ATCC 274 pleiotropically affects copper sensitivity and production of the tripyrrole secondary metabolite, prodigiosin

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Abstract The prodigiosin biosynthetic gene cluster (*pig* cluster) of *Serratia marcescens* ATCC 274 (Sma 274) is flanked by *cueR*/*copA* homologues. Inactivation of the *copA* homologue resulted in an increased sensitivity to copper, confirming that CopA is involved in copper homeostasis in Sma 274. The effect of copper on the biosynthesis of prodigiosin in Sma 274 and the *copA* mutant strain was investigated. Increased levels of copper were found to reduce prodigiosin production in the wild type Sma 274, but increase production in the *copA* mutant strain. The physiological implications for CopA mediated prodigiosin production are discussed. We also demonstrate that the gene products of *pigB–pigE* of Sma 274 are sufficient for the biosynthesis of 2-methyl-3-*n*-amyl-pyrrole and condensation with 4-methoxy-2,2′-bipyrrole-5-carboxyaldehyde to form prodigiosin, as we have shown for *Serratia* sp. ATCC 39006.

Keywords Antibiotic · Biosynthesis · Regulation · Secondary metabolite

Introduction

Over the past few years interest in prodigiosin has increased, due to its promising anti-cancer activity [17, 18]. Prodigiosin has been shown to be active against multi-drug resistant cancer cell lines [15]. Prodigiosin is also of potential clinical interest because it is reported to have anti-fungal, anti-bacterial, anti-protozoal/anti-malarial and immunosuppressive activity [5, 6, 20, 31, 32, 34].

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Prodigiosin is a red, linear tripyrrole and a member of the prodiginines, produced by some *Serratia* sp., actinomycetes and a few other bacteria [8]. Prodiginines are classical secondary metabolites only appearing in the later stages of bacterial growth [33]. Prodigiosin has no defined role in the physiology of producing strains, but it has been suggested to have a role in metabolic overflow [2, 13]. It may also be an important factor for the trypanolytic activity of *S. marcescens* [1].

Recently, the identification cloning, and sequencing, of the genes from three gene clusters responsible for the biosynthesis of prodiginines have been described [3, 11]. These were the *pig* clusters from *Serratia* sp. ATCC 39006 (*Serratia* 39006), *Serratia marcescens* ATCC 274 (Sma 274), and the *red* cluster from *Streptomyces coelicolor* A3(2) that encodes biosynthesis of undecylprodigiosin and butyl-*meta*-cycloheptyl-prodiginine in a 2:1 ratio (Fig. 1a) [3, 11, 30]. The *pig* genes of the two *Serratia* sp. only exhibit 68% sequence identity on average at the DNA level [11]. In addition, we have reported a bifurcated pathway for the biosynthesis of prodigiosin in *Serratia* 39006, which is a taxonomically ill-defined strain that does not fall into the *S. marcescens* classification [35]. The proposed bifurcated pathway is shown in Fig. 1b and culminates in the PigC-catalysed condensation of the terminal products of the two parallel pathways, 4-methoxy-2,2′-bipyrrole-5-carboxyaldehyde (MBC) and 2-methyl-3-*n*-amyl-pyrrole (MAP) [35]. In our earlier paper we assigned each individual Pig enzyme a specific role in the biosynthesis of prodigiosin, with the exception of PigK, and we also proposed a modified scheme for the biosynthesis of undecylprodigiosin which differed from the previously described biosynthetic pathway [35].

We decided to confirm that our proposed pathway for the biosynthesis of prodigiosin in the atypical *Serratia* 39006 strain also applied to the biosynthesis of prodigiosin in Sma 274, a *S. marcescens* strain. To investigate the biosynthesis of prodigiosin in the latter we generated transposon insertion mutants and

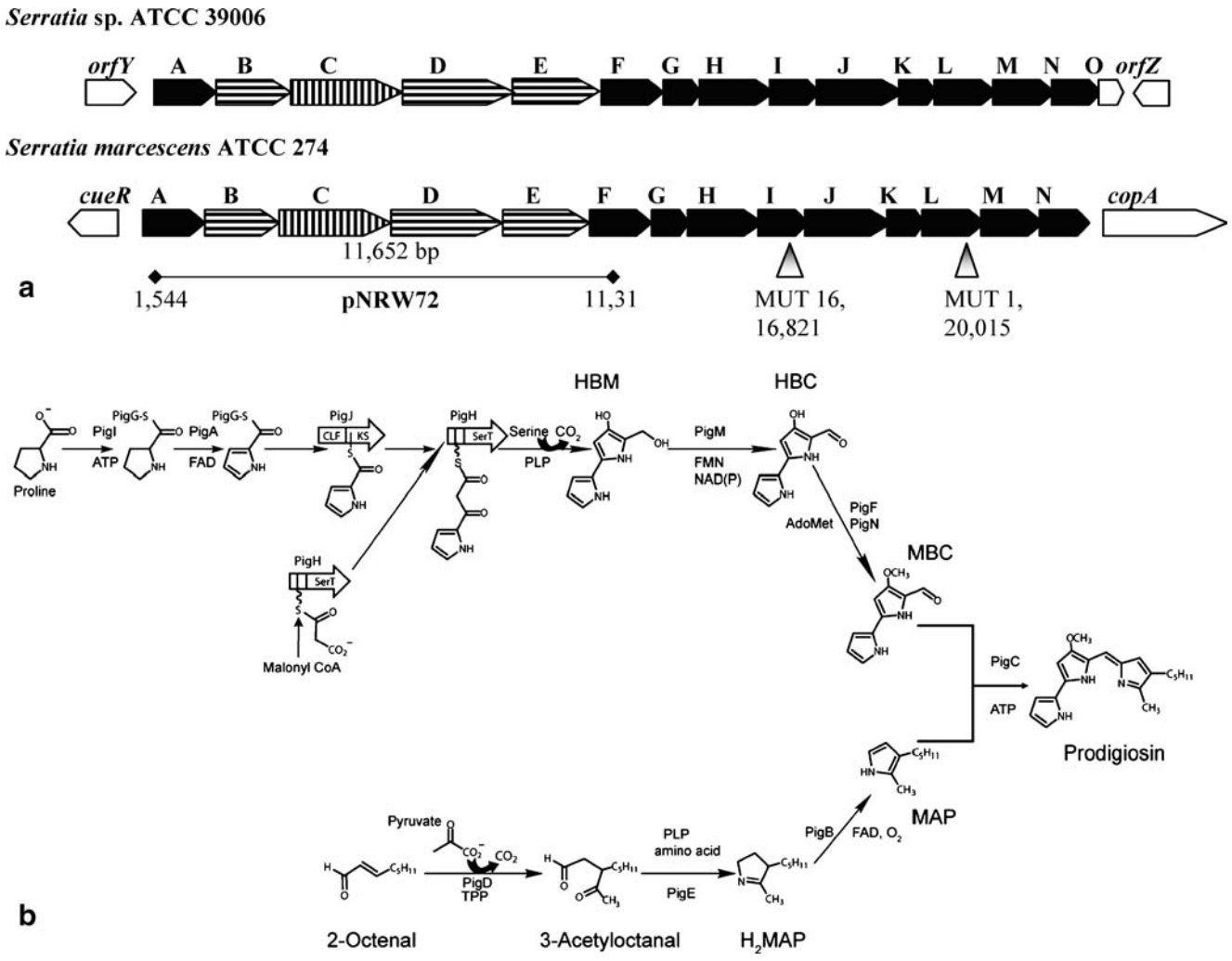

Fig. 1 **a** Schematic diagram of the prodigiosin biosynthetic clusters (*pig* cluster) of *Serratia* 39006 and Sma 274. The genes shown to direct the biosynthesis of MBC in *Serratia* 39006 are *shaded black*, and those responsible for biosynthesis of the monopyrrole are shaded with *horizontal lines*. *pigC*, which encodes the condensing enzyme (PigC), is shaded with *vertical lines*. Those genes not assigned a specific role (*pig*O) or not thought to be involved in the biosynthesis of prodigiosin are *unshaded*. Although PigK has not been assigned a specific role in the biosynthesis of prodigiosin it has been speculated that it could be a molecular chaperone involved late in the biosynthesis of MBC [35]. The position of the miniTn5 Sm/Sp transposon insertions and the fragment of the Sma 274 *pig* cluster (sufficient for MAP production in *E. coli*) cloned into pUC19 is also shown. **b** Proposed bifurcated pathway for the biosynthesis of prodigiosin by *Serratia* 39006 and the enzymes involved in each step [35]

screened for the ability of these mutants to produce specific, predicted biosynthetic intermediates.

We have previously shown that the genomic context of the *pig* clusters of *Serratia* 39006 and Sma 274 differ markedly [11]. The *pig* cluster of *Serratia* 39006 is flanked by *orfY* and *orfZ*. OrfY shows sequence similarity to an NADH oxidase, whilst OrfZ is a hypothetical protein with no known significant homologues. In Sma 274 the *pig* cluster is flanked by *cueR–copA*, as is the case for all *S. marcescens* strains we have screened [11]. In *S. marcescens* strains where the *pig* cluster is absent, the *cueR–copA* genes are adjacent and divergently transcribed [11]. CopA is a Cu(I)-translocating P-type ATPase efflux pump and a central component of copper homeostasis in *Escherichia coli* [25]. CueR is a transcriptional regulator of CopA and a member of the MerR family of transcriptional regulators [23, 29]. The conservation of the genomic context of the *pig* cluster in *S. marcescens* strains implied a regulatory connection between CueR- and CopA-mediated copper homeostasis and the biosynthesis of prodigiosin. In addition it is known that copper forms a complex with prodigiosin which can facilitate double strand DNA cleavage [22]. Therefore, in this study we investigated whether the gene predicted to encode CopA does have a role in copper homeostasis in Sma 274. We also investigated if there was a physiological connection between CopA-mediated copper homeostasis and prodigiosin biosynthesis in this *S. marcescens* strain.

Methods

Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are shown in Table 1. *E. coli* strains were cultured at 37°C and *Serratia* strains were cultured at 30°C in LB medium. Antibiotic final concentrations were as follows, unless otherwise stated: 100 μg/ml ampicillin (Ap), 50 μg/ml chloramphenicol (Cm), 50 μg/ml kanamycin (Km), 100 μg/ml streptomycin (Sm) and 10 μg/ml tetracycline (Tc).

DNA manipulations

All DNA manipulations were performed as described [27]. Genomic DNA from Sma 274 strains was extracted with the DNAeasy Tissue kit (Qiagen). DNA fragments were isolated from agarose gels using a gel extraction kit (Qiagen). Isolation of plasmids was performed with the Qiagen Miniprep kit. Oligonucleotide primers were purchased from Sigma-Genosys. Double-stranded DNA sequencing was performed at the DNA Sequencing facility, Department of Biochemistry, University of Cambridge using an ABI 3700 DNA analyser. All PCR reactions were performed using Expand high fidelity Taq polymerase (Roche).

Marker exchange of *copA*::*cat* mutant allele into the chromosome of Sma 274

To construct a *copA*::*cat* plasmid the *copA* gene was cloned into pUC18 in the following manner. Firstly, pPIG4 was digested with *Bam*HI/ *Nde*I and a 3.4 kbp fragment containing the *copA* gene was ligated, using T4 DNA ligase (Invitrogen) into pUC18 to generate plasmid pNRW30. pNRW30 was then digested with *Sph*I and a 1.132 kbp internal fragment was replaced by a *Sph*I digested *cat* (Cm^R) gene which was PCR amplified from pACYC184 to generate pNRW31. The *copA*::*cat* allele was cloned into the *Sma*I site of pKNG101 on a 3.0 kbp *Xmn*I fragment in order to create the marker exchange construct, pNRW32. Marker exchange with pNRW32 was carried out using a protocol similar to that previously described [14]. pNRW32 was introduced into *Serratia* 39006 by conjugal transfer in a three-way patch mating with *E. coli* cc118λpir (pNRW32), *E. coli* HH26 (pNJ5000) and *Serratia* 39006. pKNG101 derived plasmids have an *oriR6K* origin of replication and can only replicate in hosts expressing the π protein encoded by the *pir* gene. The cells in which pNRW32 had integrated into the chromosome of *Serratia* 39006 were selected on minimal agar containing 0.2% glucose and 100 μg/ml streptomycin (twice). A second cross-over event was selected for by plating onto minimal agar containing 10% sucrose. Excision of the plasmid DNA was facilitated by the gene product (levansucrase) of the plasmid encoded *sacB*, which catalyses the synthesis of levans (toxic compounds) in the presence of high concentrations of sucrose. Southern blotting of restriction enzyme digested chromosomal DNA with a DIG labelled probe for the Cm marker was used to confirm that the *copA* mutation was correct.

Complementation of the strain NW7

The *copA* gene was cloned on a 3.7 kbp *Ssp*I/*Bam*HI fragment from pNRW30 into the 2.9 kbp *Bam*HI/*Fsp*I plasmid backbone of pACYC177. The resulting plasmid, pNRW72, was Km^R, Ap^S. pNRW72 was used to transform the mutant strain NW7 by electroporation and the Km^R transformants were selected.

Transduction of *copA*::*cat* into Sma 274

An overnight culture of Sma 274 was grown at 30°C in 5 ml LB. The culture was centrifuged at 4,500 rpm for 10 min and the bacterial pellet was resuspended in 1 ml LB. 100 μl of a high titre ϕ3 M phage lysate grown on strain NW7 was added to the cell suspension, mixed and incubated at 30°C for 1 h. 100 μl aliquots of the phage infected cells were plated onto LB containing Km and incubated at 30°C overnight. Transductants were purified by streaking onto selective plates twice to avoid any phage carry over. 100 μl of the cells prior to the addition of the phage and 100 μl of the phage lysate were plated on LB Km as negative controls.

Prodigiosin production assay

Overnight cultures of each strain were used to inoculate 250 ml conical flasks containing 25 ml LB with the appropriate concentration of $CuSO_4$ to a final OD_{600} of 0.02. The cultures were then grown at 30°C with shaking (300 rpm) for 14 h (stationary phase) at which point prodigiosin levels and OD_{600} were measured.

Prodigiosin production was measured in the following manner. Briefly, 1 ml samples of culture were pelleted and the bacterial pellet resuspended in 1 ml acidified ethanol (4 % 1 M HCl in ethanol). The sample was pelleted again and the supernatant was transferred to a cuvette and the A_{534} was measured using a Unicam Heλios spectrophotometer. Relative prodigiosin concentration was expressed per cell ($[A_{534}/OD_{600}]\times 50$).

Cloning of the Sma 274 *pigB–pigF* genes into pUC19

An 11.6 kbp *Xho*I/*Sac*I fragment of pPIG4 containing the *pig′A–pigF* genes of Sma 274 was cloned into *Sal*I/*Sac*I digested pUC19, creating the construct pNRW73.

Table 1 List of strains and plasmids used in this study

Strain/ plasmid	Genotype/ phenotype	Source or reference
Escherichia coli		
CC118λpir	*araD*, Δ (*ara, leu*), Δ*lacZ*74, *pho*A20, *galK, thi-1*, *rspE, rpoB*, *argE, recA1*, λ *pir*	[12]
DH5α	F$^-$, *ϕ*80Δ d *lacZ*M15, Δ (*lacZYA argF*)U169, *endA1, recA1*, *hsd*R17 (r_k^- m_k^-), *deoR, thi-* 1, *sup*E44, λ$^-$ *gyr*A96, *rel*A1	GibcoBRL, Life Technologies
HH26	Mobilizing strain for conjugal transfer	[14]
Serratia		
S. marcescens 274	Wild type (Pig$^+$)	Lab stock
MUT 1	Sma 274, *pigL*::miniTn5 Sm/Sp, Sm^R	This study
MUT 16	Sma 274, *pigI*::miniTn5 Sm/Sp, Sm^R	This study
NW 7	Sma 274, *copA*::*cat*, Cm^R	This study
NW 8	*Serratia* 39006, in-frame *pigH*::57 bp	[35]
NW 13	*Serratia* 39006, in-frame *pigD*Δ	[35]
NW 14	*Serratia* 39006, in-frame *pigB*Δ	[35]
Plasmids		
pNRW30	3.4 kbp *Bam*HI/ *Nde*I fragment of pPIG4 ligated into pUC18	This study
pNRW31	*Sph*I digested Cm marker amplified from pACYC184 ligated into *Sph*I digested pNRW30	This study
pNRW32	3.0 kbp *Xmn*I fragment containing the *copA*::*cat* allele ligated into the *Sma*I site of pKNG101	This study
pNRW72	3.7 kbp *Ssp*I/*Bam*HI fragment from pNRW30 ligated into the 2.9 kbp *Bam*HI/ *Fsp*I plasmid backbone of pACYC177	This study
pNRW73	11.6 kbp *Xho*I/*Sac*I fragment of pPIG4 containing the *pig′ A pigF* (1,544–13,195)[a] genes ligated into *Sal*I/*Sac*I digested pUC19	This study
pACYC177	Low copy cloning vector, Ap^R, Km^R	[4]
pACYC184	Low copy cloning vector, Cm^R, Tc^R	[4]
pKNG101	Sm^R, *sacBR*, mobRK2, oriR6 K	[14]
pUC18	ColE1 replicon, Ap^R	[37]
pUC19	ColE1 replicon, Ap^R	[37]
pPIG4	SuperCos vector with ~41 kb insert containing the Sma 274 *pig* cluster	[11]
pTON245	pHCP19 vector, containing the *pig* cluster (24 kb) from *Serratia* 39006	[11]
pNJ5000	Mobilizing plasmid used in marker exchange, Tc^R	[9]

[a]Numbering based on Sma 274 *pig* cluster (Accession number: AJ833002)

Random transposon mutagenesis of Sma 274

A random transposon mutagenesis of Sma 274 was performed. *E. coli* SM10 carrying pUTminiTn5Sm/Sp was used to introduce the transposon into Sma 274 by conjugation as previously described [10]. Pig$^-$ mutants were identified visually.

Extraction and LC-MS analysis of biosynthetic intermediates of prodigiosin

A 5 ml overnight culture of the *pig* biosynthetic mutants was used to inoculate 200 ml of LB and the culture was grown with shaking (300 rpm) for 16–18 h at 30°C. The bacteria were pelleted by centrifugation (4,500 rpm, 10 min, 4°C). The supernatant was extracted twice with 100 ml of ether–acetone (4:1), and the cell pellet was extracted once with 100 ml of ether–acetone (4:1). The two extracts were then concentrated under reduced pressure to 5 ml, and a 300 µl sample was taken from each extract. The remaining extracts were concentrated and re-dissolved in ether–acetone (4:1) to a final concentration of 1 mg/ml. A further 300 µl sample was taken and then all samples were analysed by LC-MS as described previously [35].

Results and discussion

Biosynthesis of prodigiosin in Sma 274

A miniTn5 Sm/Sp transposon mutagenesis of Sma 274 was performed to identify *pig* biosynthetic mutants capable of producing MAP. A total of 17 white Sm/Sp transposon mutants were streaked next to MBC (*pigB*Δ

mutant; NW14) or MAP (*pigH*-H57; NW8) producing strains on peptone glycerol agar (PGM). PGM was used as this causes enhanced pigmentation and enhanced production of prodigiosin biosynthetic intermediates [19, 35]. None of the presumptive *pig* biosynthetic mutants could cross-feed each other or a strain capable of producing MAP, and hence no strains capable of producing MBC were identified. We showed previously that the *pig* cluster of *Serratia* 39006 is transcribed as a single polycistronic mRNA [28]. Therefore, this result for Sma 274 is not unexpected as a transposon insertion downstream of *pigA* would result in a polar mutation which would abolish transcription of the genes that direct MBC biosynthesis. Seven out of the seventeen white *pig* biosynthetic mutants could cross-feed, and be cross-fed by, a *pigB*Δ mutant. This result suggests that these seven strains were capable of producing MAP.

Two mutants were selected at random for further investigation. PCR amplification using sequence-specific and transposon-specific primers was used to determine the location of the transposon insertions for mutants MUT1 and MUT16. Sequence analysis of MUT1 revealed the position of the transposon insertion to be located at 20,015 bp (Sma 274 *pig* cluster numbering; Accession number: AJ833002) which is an insertion in *pigL*. Mut16 had a transposon insertion at 16,821 bp (Sma 274 *pig* cluster numbering) which corresponds to an insertion in *pigI* . LC-MS analysis of extracts of MUT1 and MUT16 identified a peak from the extract of each mutant that corresponds to MAP (Table 2). These data are consistent with our prediction based on the prodigiosin biosynthetic pathway of *Serratia* 39006 that insertions in, or downstream of, *pigI* and *pigL* would abolish MBC biosynthesis.

To confirm that *pigB–pigE* are sufficient for the biosynthesis of MAP in Sma 274, an 11.6 kbp *Xho*I/ *Sac*I fragment of pPIG4 was cloned into pUC19, creating the construct pNRW73. *E. coli* DH5α containing pNRW73 was capable of being cross-fed, and of cross-feeding, a MAP^{-} strain after growth on PGM for 2 days at 30°C (Table 2). This result indicates that *pigB–pigF* expressed in *E. coli* are sufficient for the biosynthesis of MAP, and is consistent with our observation that *E. coli* containing pNRW27 (*pigA–pigE*′ from *Serratia* 39006) is sufficient for the biosynthesis of MAP [35]. Thus, the biosynthesis of MAP in Sma 274 requires just three enzymes (PigB, PigD and PigE) as we have previously shown for *Serratia* 39006. *E. coli* containing pNRW73 could form prodigiosin when supplied with MBC indicating that PigC as in *Serratia* 39006 is the condensing enzyme. Overall these results on the biosynthesis of prodigiosin precursors in Sma 274 suggest that the assembly of the red antibiotic in *S. marcescens* is the same as in *Serratia* 39006.

Copper sensitivity of strain NW7 compared to that of wild type Sma274

The *copA* gene of Sma 274 was disrupted by the removal of a 1.1 kbp internal fragment of the gene which was replaced by a Cm^{R} antibiotic marker. The *copA*::*cat* allele was introduced into the chromosome of Sma 274 by marker exchange. The *copA*::*cat* allele was then transduced back into Sma 274 (with a clean genetic background) using ϕ3 M [10, 24].

The effect of $CuSO_4$ on the growth of NW7 in liquid LB was compared with that of wild type Sma 274 (Fig. 2). The *copA* mutant exhibits increased sensitivity to copper, at a concentration >1 mM $CuSO_4$, which was complemented by the introduction of a functional copy of *copA* supplied *in trans*. Complementation of the strain NW7 confirmed genetically that the increased sensitivity to copper was due solely to inactivated *copA* and that CopA must therefore have an important role in copper homeostasis in Sma 274. Furthermore, the ability of strain NW7 to survive at 2 mM $CuSO_4$ suggests that other genes also contribute to copper tolerance in Sma 274, as is the case in *E. coli* [7, 21, 26, 36]. In *E. coli*, other systems such as CueO (copper oxidase) and the Cus efflux complex are known to be involved in copper homeostasis [7, 21].

Growth and production of prodigiosin by Sma 274 and strain NW7 with and without the addition of 1 mM copper

The growth and biosynthesis of prodigiosin by strain NW7 was compared with that of Sma 274 in LB, and LB supplemented with 1 mM $CuSO_4$ (Fig. 3). Strain NW7 grown in LB only and Sma 274 supplemented with 1 mM $CuSO_4$ were unaffected in growth compared with that of wild type without additional copper. Compared

Table 2 Cross-feeding and LC-MS analysis of different strains

Strain	Genotype	Cross-fed by *pigB*Δ strain	Cross-feeds *pigB*Δ strain	Predominant *m*/*z* signal ($M + H^{+}$ ion) identified by LC-MS analysis	Compound corresponding to *m*/*z* signal
MUT 1	*PigL*::miniTn5 Sm/Sp	+	+	152.6	MAP
MUT 16	*pigI*::miniTn5 Sm/Sp	+	+	152.7	MAP
E. coli DH5α pNRW73	*pig′A-pigF*	+	+	ND	ND
E. coli DH5α pUC19		–	–	NA	NA

+ symbol indicates cross-feeding has occurred, – symbol shows that cross-feeding did not occur *NA* not applicable, *ND* LC-MS analysis was not performed for this strain

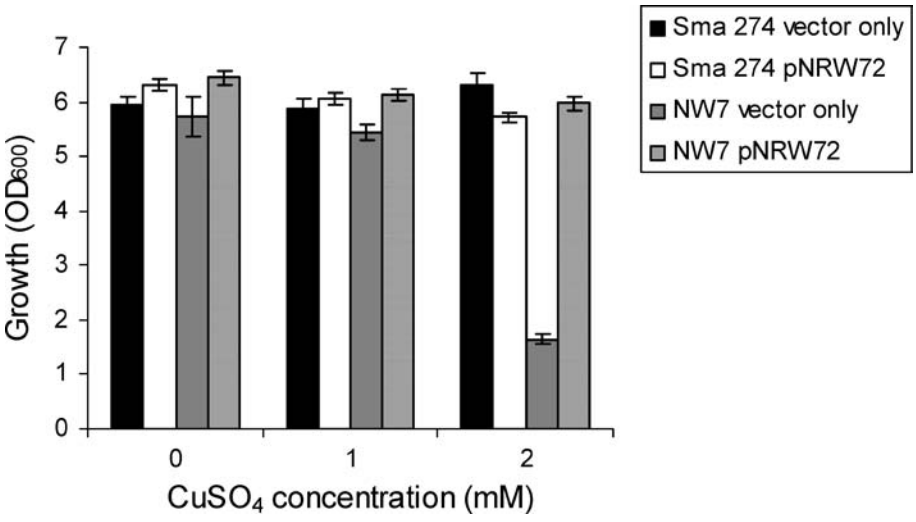

Fig. 2 Growth of Sma 274 (vector only); Sma 274 pNRW72; NW7 (vector only); and NW7 pNRW72 in LB, and LB supplemented with 1 and 2 mM $CuSO_4$ after 14 h. Complementation of the *copA* mutant strain with pNRW72 (*copA*) restored the tolerance to copper back to that of the wild type. *Error bars* show ±SEM

to wild type Sma 274, strain NW7 exhibited a very slightly reduced growth rate, when 1 mM additional copper was added to the LB, although it reached a similar final culture density (Fig. 3).

The addition of 1 mM $CuSO_4$ to LB resulted in an approximately twofold reduction in prodigiosin production in the wild type, but an approximate 1.7-fold increase in pigment production in the NW7 strain.

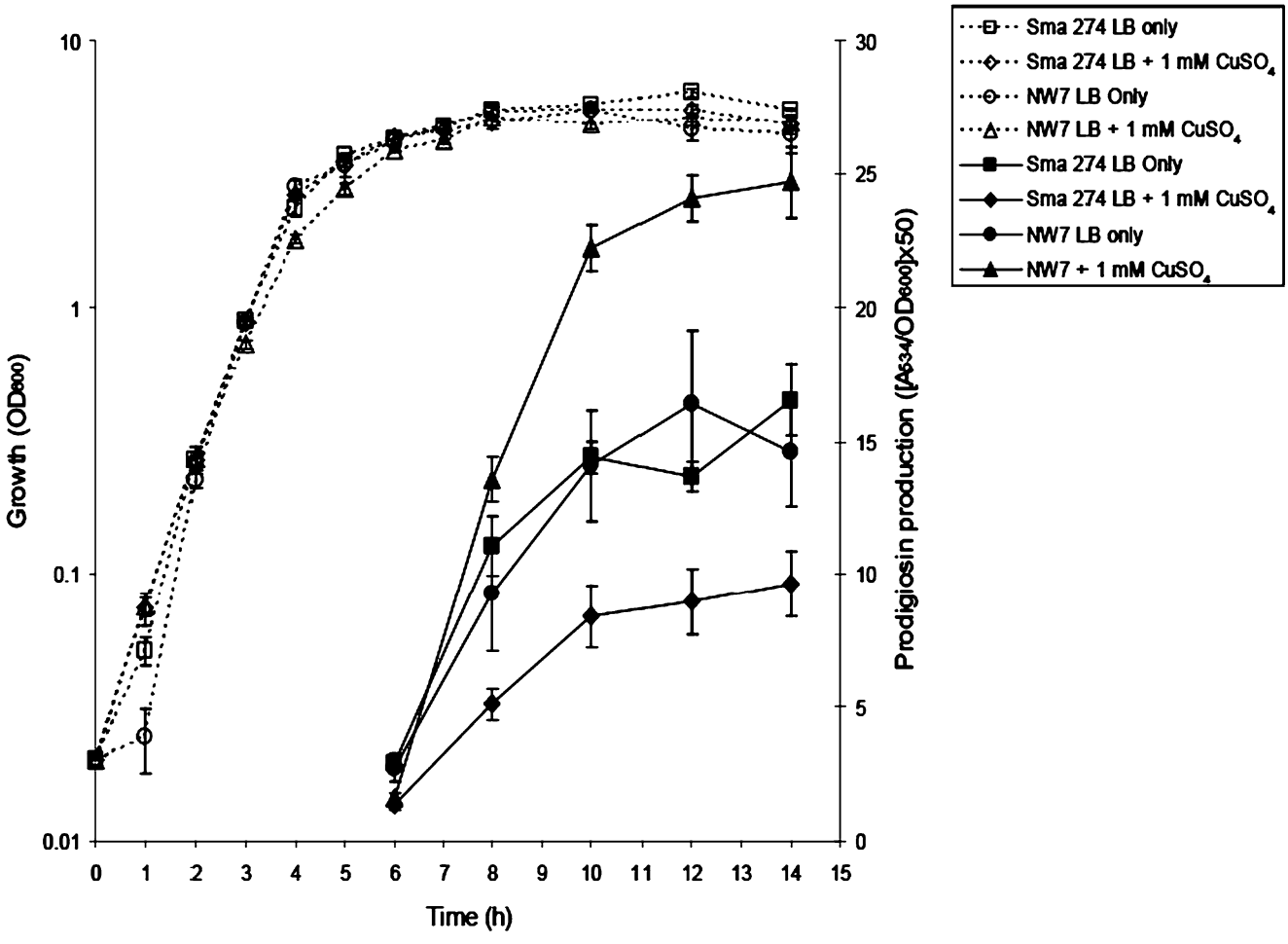

Fig. 3 Growth and production of prodigiosin by Sma 274 and the *copA* mutant strain (NW7) grown in LB with and without the addition of 1 mM $CuSO_4$. The *open symbols* and *dashed lines* represent growth (OD_{600}), and the *solid symbols* and *lines* show the production of prodigiosin. *Error bars* show ±SEM. Prodigiosin production was monitored by measuring A_{534} in acidified ethanol and prodigiosin production was expressed as ($[A_{534}/OD_{600}]\times 50$)

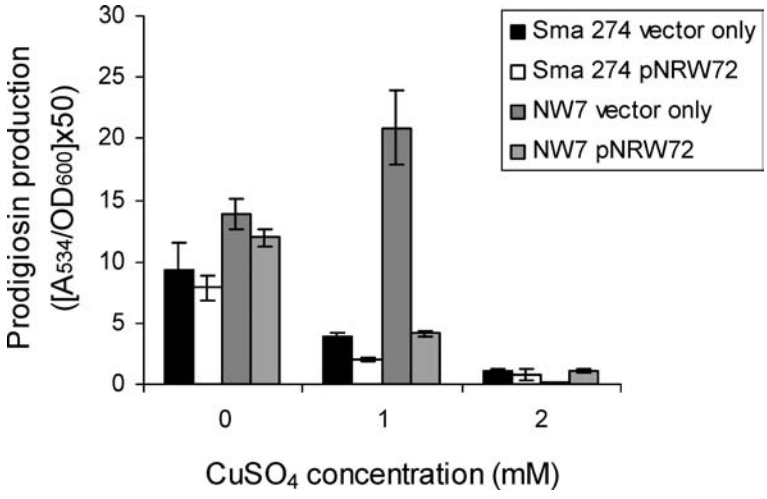

Fig. 4 Production of prodigiosin measured (A_{534} in acidified ethanol) after 14 h growth at 30°C for the following strains: Sma 274 (vector only), Sma 274 (pNRW72), NW7 (vector only) and NW7 (pNRW72). Complementation of the *copA* mutant strain with pNRW72 (*copA*) restores production of prodigiosin back to that of the wild type. *Error bars* show ±SEM

Complementation of the NW7 strain restored prodigiosin levels back to those of the wild type when the mutant was grown in LB with 1 mM $CuSO_4$ (Fig. 4). This reduction in prodigiosin production in response to sub-inhibitory concentrations of copper in Sma 274 is consistent with a previous report for another *S. marcescens* strain [16]. However, in *Serratia* 39006, the strain in which *cueR copA* do not flank the *pig* cluster, the addition of 1 mM or 2 mM $CuSO_4$ does not affect the production of prodigiosin (data not shown).

Copper has been shown to form a complex with prodigiosin which can cause oxidative cleavage of DNA [22]. Therefore, it is possible that the cell may want to reduce prodigiosin production in the presence of high levels of copper to reduce the risk of cleavage of its own DNA. However, in strain NW7 the intracellular levels of Cu(I) may not be efficiently controlled and presumably would be higher than in the wild type. It was considered a possibility that if one or more of enzymes involved in the biosynthesis of prodigiosin required copper as a cofactor the elevated copper levels might result in increased levels of prodigiosin biosynthesis. However, a database search for copper binding motifs did not identify any common copper binding motifs in any of the prodigiosin biosynthetic enzymes. Another possibility is that copper stress could act by up-regulating precursor supply thereby enhancing flux through the prodigiosin biosynthesis pathway. Alternatively as prodigiosin is capable of binding Cu(I), prodigiosin production may act to sequester excess copper. However, we constructed a *pigI–copA* double mutant which did not produce prodigiosin and the copper sensitivity of this mutant was only marginally affected (data not shown). Finally, it is possible that all of these physiological factors contribute to the overall response, but in a subtle concentration-dependent way. Further studies using gene fusions and proteomics are required to determine if the impact of copper on prodigiosin biosynthesis is reflected at the level of transcription of the *pig* genes or occurs post-translationally.

Conclusion

In this paper we show that copper stress is an another environmental stimulus which affects production of prodigiosin, causing reduced pigmentation in wild type Sma 274. However, copper did not affect prodigiosin production in *Serratia* 39006 in LB supplemented with 1 or 2 mM $CuSO_4$. Interestingly the genetic location of the *pig* cluster in the *Serratia* 39006 strain is totally different from that in *S. marscescens* strains [11]. In the latter, the *pig* cluster is always flanked by *cueR* and *copA* consistent with a role for copper homeostasis in prodigiosin production. We also demonstrate that the Sma 274 *copA* mutant strain (NW7) has increased sensitivity to copper and increased prodigiosin production. Thus CopA has a role in copper homeostasis in Sma 274 and is clearly a factor impinging on regulation of the secondary metabolite, prodigiosin.

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