J. Microbiol. Biotechnol. (2008), **18**(4), 658–662

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Effects of pH Shock on the Secretion System in *Streptomyces coelicolor* A3(2)

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Received: October 4, 2007 / Accepted: November 29, 2007

Effects of pH shock on the secretion system of *S. coelicolor* A3(2) have been investigated at a transcriptional level by using DNA microarrays. Actinorhodin secretion was observed to be highly enhanced when an acidic-pH shock was applied to surface grown cultures of *S. coelicolor* A3(2). In this culture, a gene of *actVA-orf1* encoding a putative efflux pump or transporter protein for actinorhodin was strongly upregulated. A major number of efflux pumps for other metabolites and a major number of secretion proteins for protein secretion were also observed to be upregulated with pH shock. The secretion of actinorhodin was observed to be remarkably enhanced in liquid culture as well.

Keywords: pH shock, efflux pumps, secretion proteins, *S. coelicolor*

Streptomyces species are Gram-positive, mycelia-forming soil bacteria that undergo a complex developmental program involving morphological differentiation. They are well known for the production of a wide variety of valuable compounds such as antibiotics and other pharmaceutical molecules, herbicides, industrial enzymes, *etc*. Most secondary metabolites serve as communication signals between the producer organism and other living beings and quorum-sensing signals that trigger the differentiation of the cell [7]. For this purpose, secondary metabolites are secreted to the extracellular medium through efflux proteins or pumps [11]. The efflux pump is also known to transport external toxic materials to the outside for self-protection [5].

In addition, *Streptomyces* species are attractive hosts for the extracellular production of heterologous proteins. Extracellular secretion is very important in the production

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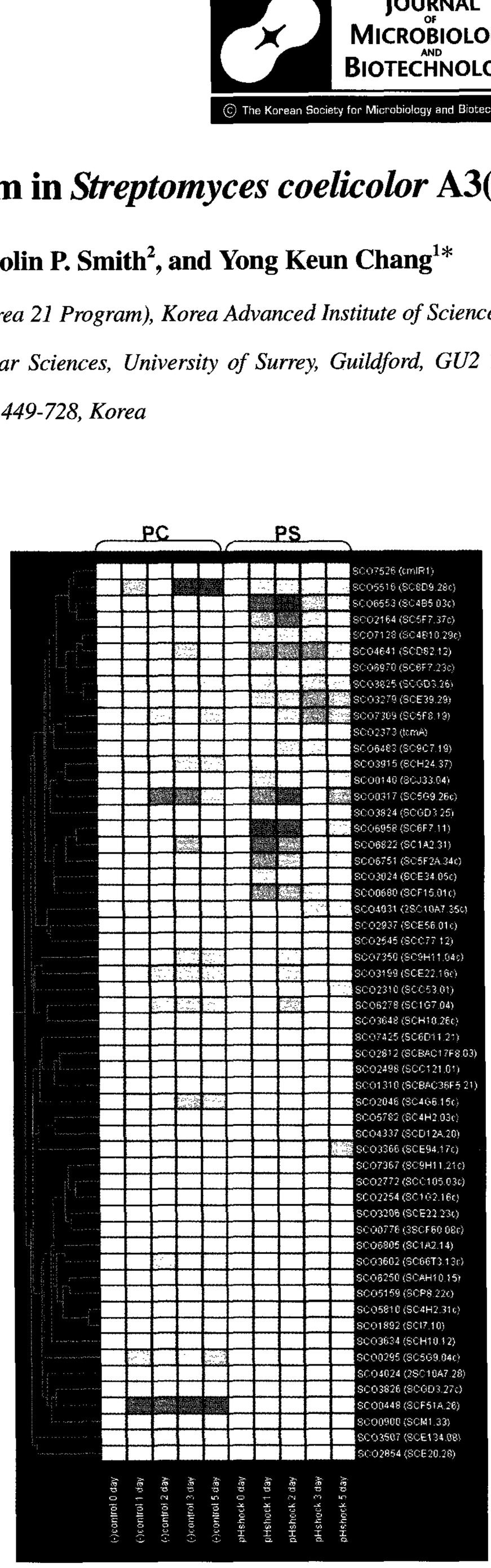

Fig. 1. DNA chip analysis results for efflux pumps.

and recovery of pharmaceutical and bioactive molecules in general. Protein secretion is mediated by secretion proteins. The interest in *Streptomyces* as a production system for valuable proteins primarily derives from its natural ability to secrete proteins into the culture broth as bioactive molecules [2, 12]. It is further based on the availability of extensive fermentation knowledge for this organism in the biopharmaceutical industry [13].

In our previous study, actinorhodin production and secretion were observed to be highly enhanced when an acidic-pH shock was applied to surface grown cultures of *S. coelicolor* A3(2) M145 (ATCC BAA471). This intrigued us about the pH shock effect on the secretion system.

Here, a transcriptional-level study was performed by using DNA microarrays to investigate the effects of pH shock on the secretion system of *S. coelicolor* A3(2) for various bioactive molecules such as antibiotics and proteins. Samples were taken from 2 different types of culture: pH-shocked culture (PS) and pH-controlled culture (PC), as previously reported [10]. In PS, cells were grown on a cellophane film placed on supplemented minimal medium solid (SMMS) [8] with an initial pH of 7.2 for 2 days before being transferred to a new plate with a pH of 4, and then incubated 7 more days. No TES buffer was used. In PC, cells were grown on a cellophane film places on SMMS with TES buffer to suppress spontaneous pH change, for 9 days with no transfer to a new plate. For RNA sample preparation, cells were washed with water containing diethyl pyrocarbonate, and resuspended in RNAprotect Bacteria Reagent (Qiagen) for 5 min. An RNeasy Midi kit (Qiagen) was used for RNA isolation according to the manufacturer's instructions. Contaminant DNA in the sample was eliminated by using RNase-free DNase (Qiagen). The total RNA was quantified using a NanoDrop ND-1000 (Nanodrop, U.S.A.). RNA integrity was assessed using a Bioanalyzer (Agilent Technologies). The methods used for DNA microarray analysis are detailed at http://www.surrey.ac.uk/SBMS/Fgenomics/Microarrays/ and in the previous report [10].

Cultures were carried out in a 5-l jar fermentor (Korea Fermentor Co., Incheon, Korea) with 2.5 l working volume to investigate pH shock effect on the secretion of actinorhodin in liquid culture, which is the most common method for bioproducts production in general. Seed culture of 250 ml grown in a liquid SMM for 36 h was inoculated into the fermentor containing 2,250 ml of the same medium before being cultivated for 7 days at 28°C. The aeration rate was 1.0 vvm. Dissolved oxygen concentration was maintained over 20% of saturation by manipulating the agitation speed. An acidic pH shock was applied at 65 h by suddenly dropping the pH to 4.0 by using 1 N HCl. The pH was then maintained for 6 h at 4.0 before being artificially increased to 7.0 by using 1 N NH_4OH. Thereafter, it was maintained at this level until the end of the culture. Cell concentration was measured in dry cell weight (DCW). Glucose concentration was measured by using a Glucose Analyzer (YSI 2700, U.S.A.). The intracellular and extracellular amounts of actinorhodin produced were separately measured following the procedures previously reported [4, 9].

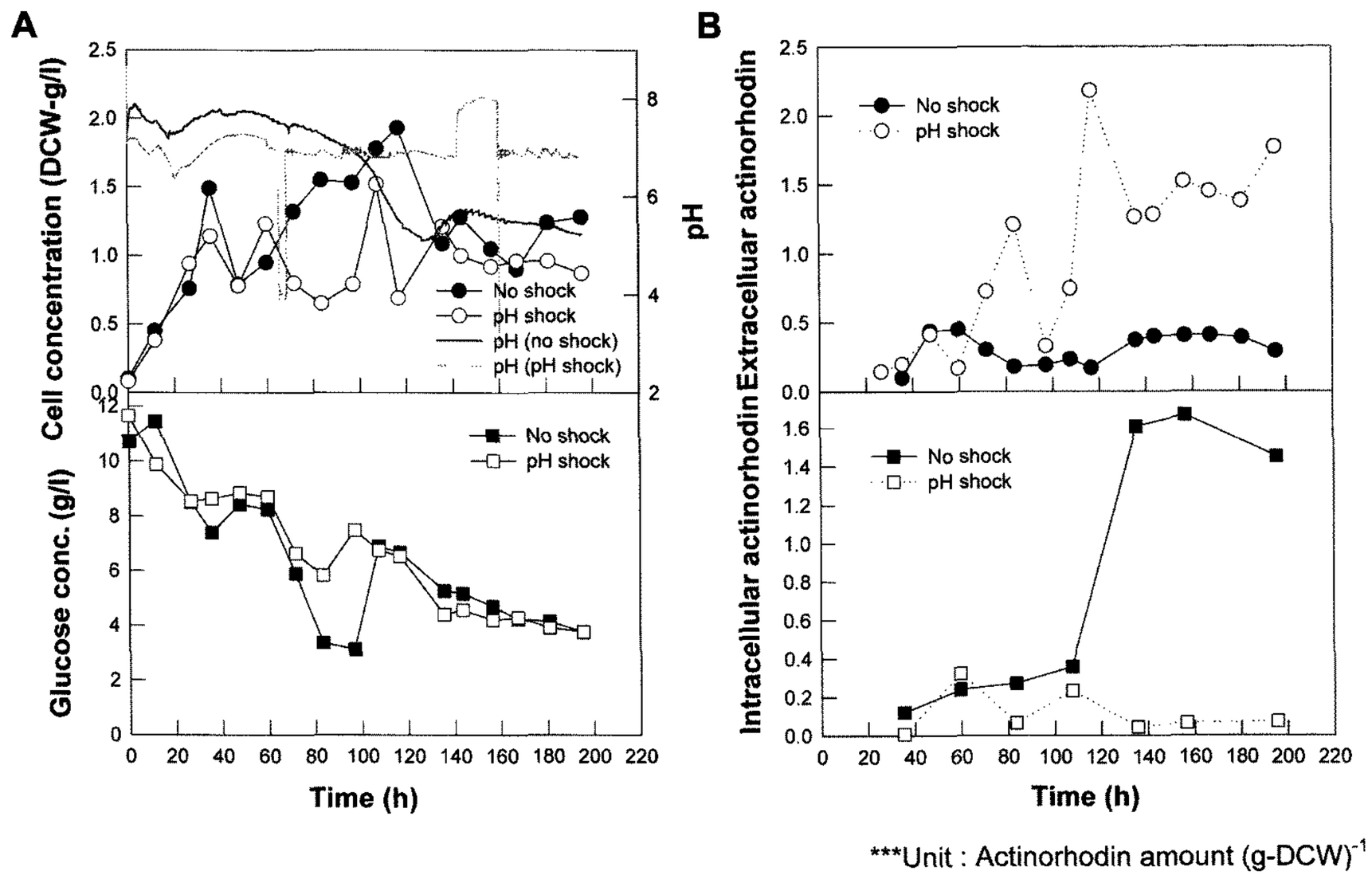

Fig. 2. pH shock effects on actinorhodin production and secretion in bioreactor culture.

As presented in our previous report, actinorhodin production and secretion were observed to be highly enhanced when an acidic-pH shock was applied to surface grown cultures of *S. coelicolor* A3(2) [10]. In PS, 72% of actinorhodin produced (1.41/g-DCW out of 1.95/g-DCW) was secreted, whereas in the culture with no pH shock (PC), only 33% (0.01 out of 0.03) was secreted. A gene of *actVA-orf1* encoding a putative actinorhodin transporter protein having the function of the efflux pump was strongly upregulated in PS. ActVA-ORF1 is known to play a positive role in actinorhodin secretion together with ActII-ORF2, another efflux pump for actinorhodin secretion [3, 11]. However, the expression of *actII-orf2* did not show any notable difference between PS and PC.

In the present study, we investigated the effects of pH shock on the secretion system for metabolites and secreted proteins in general, whereas only secretion proteins specific to actinorhodin were examined in the previous study. To date, 56 efflux pumps, although putative, have been found in *S. coelicolor* A3(2) in addition to ActVA-ORF1 and ActII-ORF2, mentioned earlier as two actinorhodin-specific efflux pumps. The metabolite that each of the

Table 1. DNA chip analysis results for efflux pumps.

SCO number	Expected function	Up and down ratio	Homolog	e-value
SCO0140	Putative *merR*-family transcriptional regulator	–	BmrR, transcription regulator	5e-41
SCO0295	Putative transmembrane efflux protein	+	Sugar (and other) transporter	2e-04
SCO0448	Putative transmembrane efflux protein	+	Superfamily: MFS general substrate transporter	4.5e-15
SCO0680	Putative transmembrane efflux protein	–	AraJ, arabinose efflux permease	5e-04
SCO0776	Putative integral membrane protein	+	Cation_efflux	2e-28
SCO0900	Putative transmembrane efflux protein	+	Bacterial protein of unknown function	8e-05
SCO1310	Putative cation efflux system protein	+	Cation_efflux	2e-28
SCO1892	Putative integral membrane efflux protein	+	Superfamily: MFS general substrate transporter	6.5e-34
SCO2046	Putative integral membrane efflux protein	+	Superfamily: MFS general substrate transporter	2.3e-30
SCO2164	Putative integral membrane efflux protein	–	ACR_transporter, AcrB/AcrD/AcrF family	1e-125
SCO2254	Putative transmembrane efflux protein	+	Sugar (and other) transporter	2e-04
SCO2545	Putative transmembrane efflux protein	–	Superfamily: MFS general substrate transporter	1.3e-20
SCO2772	Putative membrane transport protein	+	Cation_efflux	5e-32
SCO2812	Putative drug efflux protein	+	Superfamily: MFS general substrate transporter	8.3e-39
SCO2937	Putative transmembrane transport protein	-	Sugar (and other) transporter	2e-07
SCO3199	Putative transmembrane efflux protein	+	Sugar (and other) transporter	2e-07
SCO3206	Putative transmembrane efflux protein	+	Superfamily: MFS general substrate transporter	3e-75
SCO3279	Putative integral membrane efflux protein	–	Na_H_Exchanger	2e-08
SCO3366	Putative exporter	+	Fungal trichothecene efflux pump	1e-21
SCO3507	Putative integral membrane efflux protein	+	Bacterial protein of unknown function	2e-25
SCO3602	Putative transmembrane transport protein	+	KefB, Kef-type K+ transport systems	3e-11
SCO3648	Putative transmembrane efflux protein	–	Sugar (and other) transporter	2e-08
SCO3826	Putative ion channel membrane protein	+	TrkA-N domain	1e-15
SCO4024	Putative integral membrane efflux protein	+	AraJ, Arabinose efflux permease	2e-05
SCO4031	Putative integral membrane transport protein	+	TRI12, Fungal trichothecene efflux pump	6e-08
SCO4337	Putative integral membrane efflux protein	+	Bacterial protein of unknown function	8e-34
SCO5159	Putative integral membrane transport protein	+	AraJ, Arabinose efflux permease	2e-06
SCO5782	Putative transmembrane transport protein	+	KefB, Kef-type K+ transport systems	2e-21
SCO5810	Putative transmembrane efflux protein	+	Superfamily: MFS general substrate transporter	4.3e-79
SCO6278	Putative integral membrane transport protein	+	Superfamily: MFS general substrate transporter	1.9e-69
SCO6483	Putative efflux protein	–	Sugar (and other) transporter	8e-11
SCO6553	Putative integral membrane efflux protein	–	AraJ, Arabinose efflux permease	2e-04
SCO6751	Putative efflux protein	–	Cation efflux family	5e-46
SCO6958	Putative membrane protein	–	K-efflux system protein, PhaF	2.8e-05
SCO6970	Putative membrane transport protein	–	TRI12, Fungal trichothecene efflux pump	5e-16
SCO7128	Putative transmembrane efflux protein	–	Sugar (and other) transporter	1e-08
SCO7350	Putative membrane efflux protein	+	Superfamily: MFS general substrate transporter	3.7e-34
SCO7367	Putative membrane efflux protein	+	ArsB_permease, Anion permease ArsB	5e-39
SCO7425	Putative multidrug-efflux transporter protein	–	Sugar (and other) transporter	3e-07
SCO7526	Chloramphenicol resistance protein	–	AraJ, Arabinose efflux permease	3e-32

efflux pumps are related to has not been identified yet. Fig. 1 and Table 1 show that 25 genes among 56 genes encoding the efflux pump were upregulated with pH shock, whereas 16 genes were downregulated. The rest of the 15 genes showed no significant differences. Considering that the number of upregulated genes was significantly larger than that of downregulated ones, the secretion system overall seemed to be activated by pH shock. No specific and detail discussion is possible at the moment owing to lack of information on the role of each efflux protein. Specifically, SCO4337, SCO3602, SCO5159, SCO5810, SCO0295, SCO0448, and SCO0900 were highly upregulated, and SCO6970 and SCO6751 were strongly downregulated.

Table 2 shows the results from DNA chip analysis of secretion proteins (proteins mediating protein secretion). Fifteen genes encoding secretion proteins listed for *S. coelicolor* A3(2) in the GOdatabase (http://www.godatabase.org) were examined. Among them, 8 genes (53%) were upregulated, whereas only 3 genes (20%) were downregulated with the pH shock. In particular, SCO1515, SCO1516, SCO4527, and SCO452 were highly upregulated. In the case of *S. lividans*, two main secretion mechanisms are Sec (from "secretion") and the twin-arginine translocation (Tat) pathways [1, 6]. The mechanism of Tat-dependent protein secretion is reported to be completely different from that of Sec-dependent secretion. In this study, the only Tat-dependent secretion protein in *S. coelicolor* A3(2), that is, *tatB* (SCO5150), was observed not to be affected by pH shock. However, the expressions of Sec-dependent secretion proteins were observed to be upregulated by pH shock. Among 5 *sec* genes, *secF* (SCO1515), *secY* (SCO4722), and *secD* (SCO1516) were upregulated and *sec*G (SCO1944) was downregulated by pH shock, whereas *sec*E (SCO4646) showed no difference. Moreover, putative SecDF protein-export membrane protein (SCO6160) was upregulated.

To confirm the effect of secretion enhancement by pH shock in liquid culture, pH shock was applied to a bioreactor culture (Fig. 2). When a pH shock was applied as described earlier, the cell growth was severely inhibited. The total amount of actinorhodin produced was comparable to that of the control culture with no pH shock. However, the amount of extracellular actinorhodin in PS was about 4 times higher than the controlled culture. The results from both surface grown cultures and liquid cultures clearly showed that an artificially enforced pH-drop-and-recovery could enhance actinorhodin secretion.

These results show how an acidic-pH shock affects the expression profiles of genes related to efflux pumps and secretion proteins in the transcriptional level. It can be useful information for scientists and engineers working in the area of biopharmaceuticals production.

Acknowledgments

This work was supported by grant No.R01-2006-000-10860-0 from the Basic Research Program of the Korea Science & Engineering Foundation. We thank Giselda Bucca and Vassilios Mersinias for assisting J. Y. Song in microarray-related techniques during his visit at the University of Surrey.

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Table 2. DNA chip analysis results for secretion proteins.

SCO number	Expected function	Up and down ratio	Homolog	e-value
SCO1515	*secF*, protein-export membrane protein	+	SecD_SecF, Protein export membrane protein	6e-68
SCO1516	*secD*, protein-export membrane protein	+	SecD_SecF, Protein export membrane protein	1e-04
SCO1944	*secG*, protein-export membrane protein	–	SecG protein	1.1e-12
SCO3554	Putative membrane integral protein	–	Hypothetical protein from *Mycobacterium tuberculosis*	4.9e-11
SCO3555	Putative membrane integral protein	+	Hypothetical protein	4.5e-12
SCO4528	Putative membrane integral protein	+	-	-
SCO4722	*secY*, preprotein translocase SecY subunit	+	Eubacterial secY	1e-99
SCO5011	Putative membrane integral protein	+	Bacterial type II secretion system protein F domain	3e-07
SCO6160	Putative SecDF protein-export membrane protein	+	SecD_SecF, Protein export membrane protein	3e-48
SCP1.171	Putative membrane integral protein	–	Putative integral membrane protein	3e-16

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