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GENOMICS AND PROTEOMICS

pH shock induces overexpression of regulatory and biosynthetic genes for actinorhodin production in *Streptomyces coelicolor* A3(2)

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Abstract Actinorhodin production is markedly enhanced when an acidic pH shock is applied to a surface-grown culture of *Streptomyces coelicolor* A3(2). For an in-depth study of this phenomenon, transcriptional analyses using DNA microarrays and reverse transcription polymerase chain reaction and proteomic analysis were performed. Investigated were expression levels of the regulators and enzymes responsible for signal transduction and actinorhodin biosynthesis and enzymes involved in some major metabolic pathways. Regulators PkaG, AfsR, AfsS and/or another unidentified regulator and ActII-ORF4, in sequence, were observed to be activated by pH shock. In addition, a number of genes associated with actinorhodin production and secretion and the major central metabolic pathways investigated were observed to be upregulated with pH shock. Fatty acid degradation was particularly promoted by pH shock, while fatty acid biosynthesis was suppressed; it is envisaged that this enriches the precursor pool (acetyl-CoA) and building blocks for actinorhodin biosynthesis. Furthermore, glucose 6-phosphate dehydrogenases, initiating the pentose phosphate pathway, were highly activated by pH shock, enriching the reduced nicotinamide adenine dinucleotide phosphate (NADPH) pool for biosynthesis in general. It is deduced that these metabolic changes caused by pH shock have positively contributed to the stimulation of actinorhodin biosynthesis in a concerted manner.

Keywords pH shock · Actinorhodin production · Signal transduction · *S. coelicolor*

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Introduction

A major proportion of pharmaceutically important secondary metabolites, including antibiotics and anticancer agents, are produced by actinomycetes. For this reason, many laboratories are focusing their attention on actinomycetes to investigate the metabolic and genetic regulation of secondary metabolite production, their complicated biosynthetic mechanisms and ways of enhancing productivity. Studies of this kind include DNA microarray analysis to identify the global regulatory pathways in the production of secondary metabolites and in programming morphological differentiation (Huang et al. 2001) and proteomic analysis of secondary metabolism pathways (Hesketh et al. 2002). Although through this type of research a large amount of data has been generated, there is little useful information directly connected to the biosynthesis of particular secondary metabolites. Most of the published studies on the enhancement of secondary metabolites have focused on the overexpression of one positive regulator or by the deletion of one negative regulator at a time (Bruheim et al. 2002; Hu et al. 2002), resulting in a relatively low enhancement of

production. This is due to the fact that it is difficult to change the whole metabolic system just by manipulating one or two targeted genes because actinomycete strains have a complicated secondary metabolism. To overcome this limitation, a more global and potent methodology is necessary.

An alternative is the application of an external stimulus or stress, for example, an abrupt change in the environmental conditions such as salt shock, oxidative stress, or pH shock. It has been reported that regulators involved signal transduction networks are sensitive to external stimuli (Beppu 1995; Horinouchi 2003; Horinouchi and Beppu 1992). Methylenomycin production by *Streptomyces coelicolor* A3(2) was reported to be promoted by an acidic pH shock in a bioreactor culture (Hayes et al. 1997). In another example with *S. coelicolor* A3(2), undecylprodigiosin production was enhanced by salt shock (Sevcikova and Kormanec 2004). Proteins belonging to cold shock protein (csp) family were reported to be involved in the production of tetracycline by *S. aureofaciens* (Mikilik et al. 1999). In one of our previous studies, a more than sevenfold enhancement in kasugamycin production in a bioreactor culture of *S. kasugaensis* was observed when an artificial pH drop and recovery, a 'pH shock', was introduced (Kim et al. 2000, 2001a).

In the present report, an acidic pH shock was applied to surface grown cultures of *S. coelicolor* A3(2) to observe how effectively this environmental stress would enhance actinorhodin production and to investigate the changes in transcript and protein levels. We used DNA microarrays and reverse transcription polymerase chain reaction (RT-PCR) to determine the transcript levels of the regulators and enzymes responsible for signal transduction and actinorhodin biosynthesis and the transcriptional levels of the enzymes involved in some major metabolic pathways. In the proteomic analysis, the levels of proteins involved in actinorhodin biosynthesis and those major metabolic pathways were measured. To the best of our knowledge, this is the first report of a combined transcriptomic and proteomic approach to investigating the enhancement of secondary metabolite biosynthesis in an actinomycete.

Materials and methods

Strain, media, and culture conditions

S. coelicolor A3(2) M145 (ATCC BAA471) was grown on a supplemented solid minimal medium (SMMS) at 28°C (Kieser et al. 2000); for the pH shock cultures, TES buffer was omitted from the SMMS. A spore stock in 20% glycerol stored at −70°C was used for inoculation of cellophane film-coated SMMS plates. An inoculum of 2.8×10^7 spores was used for every culture.

pH shock implementation An artificial *acidic pH shock* was applied to the surface grown culture. The experimental strategy is shown in Fig. 1. SMMS *with no TES buffer* was used to eliminate the buffering effect and thus to allow spontaneous pH changes during the culture. The initial pH was 7.2. Cells were cultivated for 2 days before being transferred to SMMS with a pH of 4. At the moment of transfer, the pH of the medium was about 5.3. The transferred mycelium was incubated for another 7 days (9 days in total). The culture with no transfer to a new medium was designated the pH-noncontrolled culture (PNC). For the pH-controlled culture (PC), cells were cultivated on normal SMMS to suppress pH changes. The pH of the solid media was measured by using TEST PAPER (Toyo Roshi Kaisha, Japan).

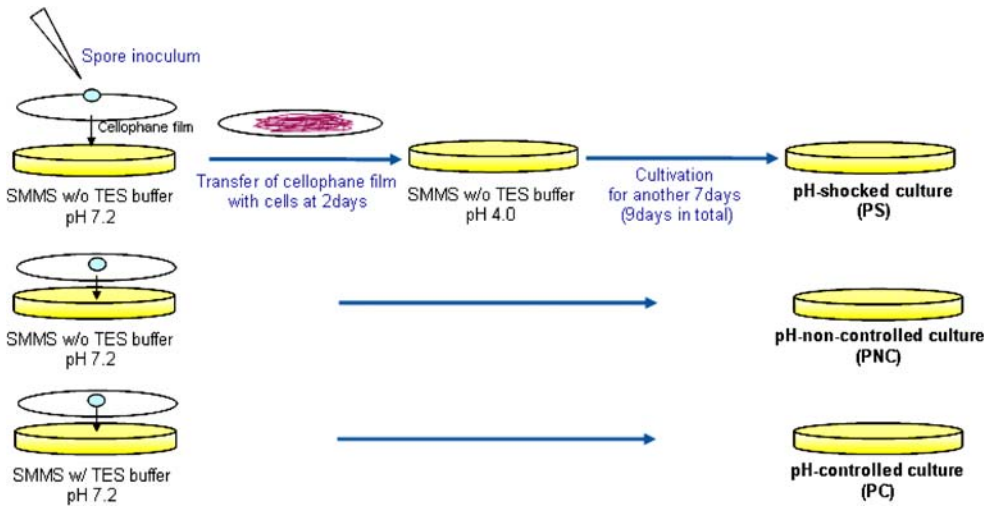


Fig. 1 Experimental design for solid culture to investigate effects of acidic pH shock

Analysis

Cell and actinorhodin concentrations

Cell concentration was measured in dry cell weight (DCW). Cells collected off the cellophane film were washed with phosphate buffer. The washed cells were dried at 80°C for 24 h and then weighed at room temperature. The intracellular and extracellular amounts of actinorhodin produced were separately measured following the procedures previously reported (Bystrykh et al. 1996). For the analysis of intracellular actinorhodin, 20 mg of dried cells was extracted with 5 ml of chloroform in a test tube for 30 min at room temperature. Then, 5 ml of 1 N NaOH was added. The mixture was vortexed and spun in a microcentrifuge for 15 s. The resulting aqueous phase contained actinorhodin, having a blue color at an alkaline pH of 12. The optical density of the aqueous phase was determined at 615 nm. For the analysis of actinorhodin secreted into agar, the agar was heat-melted before optical density measurement (pH 12 and 615 nm).

Transcriptional analysis

RNA sample preparation Cells were washed with water containing diethyl pyrocarbonate and resuspended in RNAprotect™ Bacteria Reagent (Qiagen) for 5 min. After centrifugation, the cell pellet was stored at −70°C. 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, USA). RNA integrity was assessed using a Bioanalyzer (Agilent Technologies).

DNA microarray analysis The methods used are detailed at http://www.surrey.ac.uk/SBMS/Fgenomics/Microarrays/. Briefly, for RNA labeling, 15 μg of total RNA and 1.7 μl of random primer (Invitrogen) were mixed and incubated for 10 min at 70°C, snap cooled on ice, and then 6 μl of 5× First strand buffer, 3 μl of 100 mM DTT, 0.6 μl of dNTP (25 mM each dA/G/TTP, 10 mM dCTP), 2 μl of a Super Script II (Invitrogen), and 1.5 μl of Cy3-dCTP (Amersham Biosciences) were added to make a final volume of 30 μl. The mixture was incubated for 10 min at 25°C in the dark, and further incubation was done for 120 min at 42°C in the dark. Ten microliters of 1 N NaOH was added to the incubated mixture. After incubation for 10 min at 70°C, 10 μl of 1 N HCl was added for RNA denaturation.

For genomic DNA labeling, 3.5 μg of genomic DNA, 1 μl of random primer (Invitrogen), and distilled water were mixed to make a final volume of 41.5 μl. The mixture was heated to 95°C for 5 min and snap cooled on ice, and then added with 5 μl of 10× Klenow buffer, 1 μl of dNTP (5 mM each dA/G/TTP, 2 mM dCTP), 1.5 μl of Cy5-dCTP (Amersham Bioscience), and 1 μl of Klenow fragment (New England Biolabs, UK). Oligonucleotide DNA microarrays representing 98% of *S. coelicolor* ORF's (fabricated in the Functional Genomics Laboratory, University of Surrey, UK) were used (http://www.surrey.ac.uk/SBMS/Fgenomics/Microarrays/html_code/Oligo_array.html). Hybridization was carried out using a Pronto! Universal Hybridization kit (Corning, USA) according to the supplier's instruction. Equal amounts of Cy3-labeled and Cy5-labeled samples were mixed and dried completely using a vacuum centrifuge. The dried sample was redissolved in 40 μl of a Pronto! Universal Hybridization Solution for long oligonucleotides. The resulting hybridization solution was heated to 95°C for 5 min and applied to a microarray. The hybridized microarray was incubated for 16 h at 42°C and then scanned with an Affymetrix 428 scanner (Affymetrix, USA). The 16bit-TIFF image obtained was analyzed using BlueFuse 2.0 (BlueGnome, Cambridge, UK) and GeneSpring GX 7.3.1 (Agilent Technologies).

RT-PCR analysis RT-PCR was carried out by using the primer pairs listed in Table 1. The primer sequences based on BLAST searches of the *Sanger Center* database. The negative control was carried out with Taq DNA Polymerase (Promega) in the absence of reverse transcriptase to confirm that the amplified product was not from chromosomal DNA. Transcript detection analysis was carried out by using a SUPERSCRIPT One-Step RT-PCR kit (Invitrogen) with 0.25 ìg of total RNA as template. Dimethyl sulfoxide (5% *v*/*v*) was added to the reaction mixture. Reaction conditions were as follows: first strand cDNA synthesis, at 45°C for 30 min; initial denaturation, at 94°C for 2 min; and amplification, 30 cycles of 95°C for 30 s, 50–60°C for 30 s and 72°C for 30 s. ScoF, a cold shock protein of *S. coelicolor,* was used as the internal standard (Fig. 3a).

Table 1 Oligonucleotide primers used for RT-PCR

Genes	Oligonucleotides	Product length
pkaG	F: 5′-gtg tca gcg ctg gag cca gac gac-3′ R: 5′-cgc gaa ggc cag cac cgc gcc gag-3′	617 bp
afsK	F: 5′-gtg gtg gat cag ctg acg cag cac-3′ R: 5′-cga ccg ggt tgg cgc cgt gga agg-3′	662 bp
afsR	F: 5′-ggc aag ggc ctc ttc cac cag gc-3′ R: 5′-cgt gct gct cgt aga ggt tga gc-3′	500 bp
afsS	F: 5′-cta ctt gcc gtc gcc gtc cag gtc-3′ R: 5′-gag cga caa gat gaa gga cgc gg-3′	192 bp
actII-orf4	F: 5′-tca tcg agg agt tgt ggg cgg ac-3′ R: 5′-ccg ccg gag att ccg ata cga tt-3′	542 bp

Proteomic analysis

Cells were suspended in TE buffer containing COCKTAIL (Roche) and then disrupted with a cell homogenizer. Cell debris was removed by centrifugation at 4°C for 20 min. Samples containing 100 μg proteins were prepared from the supernatant for the subsequent 1D and 2D analyses. The samples were stored at −70°C.

1DE ESI-MS/MS analysis Protein samples were treated by 1D sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands were excised and digested in-gel with trypsin. To identify the major proteins in each protein band on the gel, tryptic peptides were submitted for Electrospray Ionization Quadrupole-Time of Flight instrument (ESI-Q-TOF MS). Peptide mass fingerprints were analyzed by using the MASCOT (http://www.matrixscience.com). The bacterial database of National Center for Biotechnology Information (NCBI) was used as reference for the identification of target proteins.

2D-gel electrophoresis For the first-dimension isoelectric focusing, 18 cm IPG strips pH 4–7 (Amersham Biosciences) were rehydrated overnight (10 h) in IEF buffer containing 6.4 mg DTT and 2% ampholytes. Then separation was performed for 110,000 V-h with a maximum voltage of 5,000 V. After the isoelectric focusing, the IPG strips were equilibrated for the second dimension separation, for 15 min in IPG equilibration buffer (50 mM Tris, pH 6.8, 6 M urea, 30% glycerol, 1% SDS, and 0.01% bromophenol blue) supplemented with 80 mM DTT, and then for 15 min in IPG equilibration buffer supplemented with 135 mM iodoacetamide. Proteins were separated on 12.5% gels. Gels were then stained with a silver staining kit (Amersham Biosciences) and scanned by using a UMAX power look 1120 scanner (UMAX, Taiwan). Image analysis

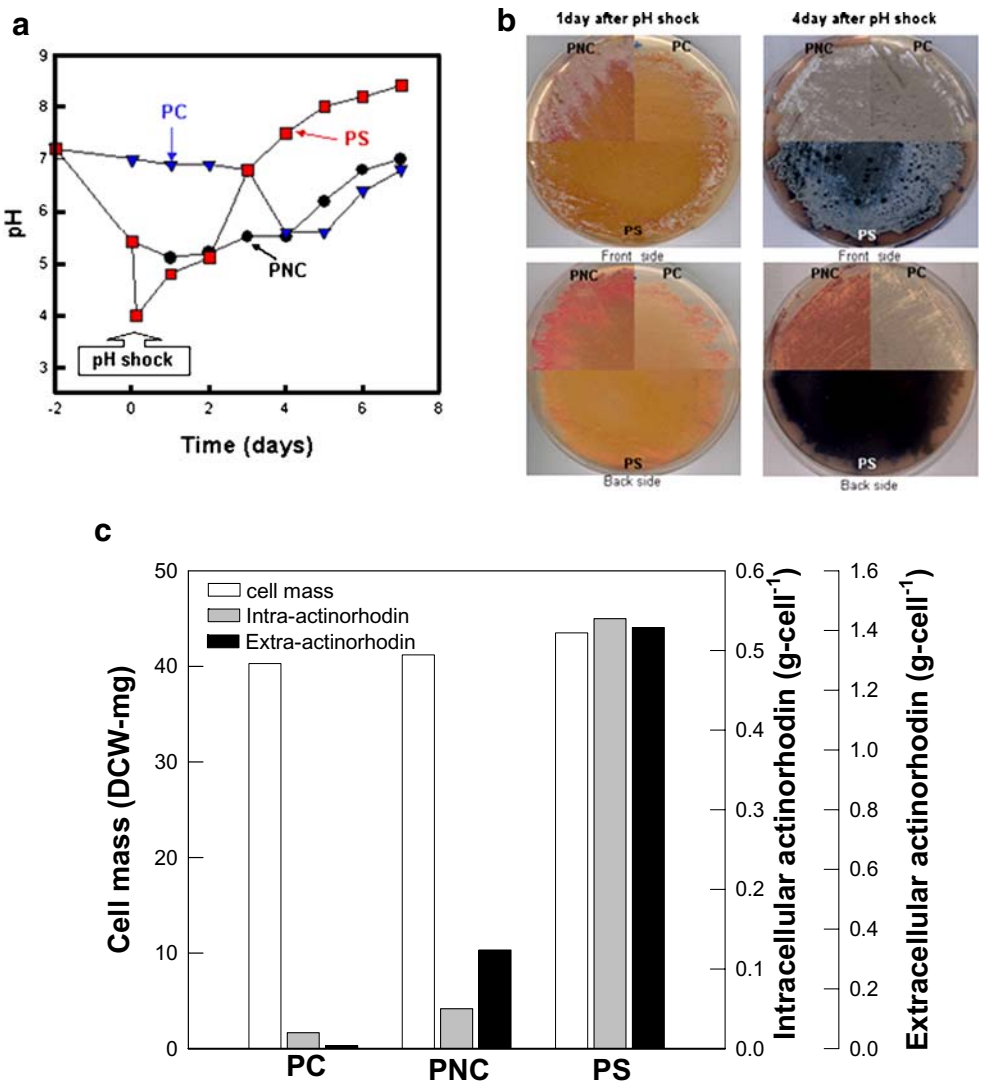


Fig. 2 **a** pH profiles, **b** pictures of culture plates, and **c** cell mass and actinorhodin production: *PNC*, pH-noncontrolled culture (without TES buffer); *PC*, pH-controlled culture (with TES buffer); and *PS*, pH-shocked culture

was performed by using Phoretix 2D Expression (NonLinear Dynamics, UK). Protein identification was performed by using a Matrix Assisted Laser Desorption/Ionizing-Time of Flight (MALDI-TOF) mass spectrometry system (Voyager DE-STR, PE Biosystem, Framingham, MA) in NICEM (National Instrumentation Center for Environmental Management).

Results

pH changes during cultivation of *S. coelicolor*

Figure 2 shows pH profiles and pictures of a pH-shocked culture (PS) and the PNC and PC. In the PNC, the pH initially dropped and then recovered to the original level. In the culture with pH shock, the pH began to increase immediately after the pH shock to reach a final level of about 8.5. In the PC, pH change was suppressed until 5 days of cultivation. The pictures in Fig. 2 clearly show that more actinorhodin, a blue-pigmented antibiotic, was produced in the order of PS, PNC, and PC.

Effects of pH shock on cell growth and actinorhodin production

In Fig. 2c, the amount of actinorhodin produced in each culture was expressed in optical density at 615 nm scaled by cell mass. When pH was artificially changed to 4, the highest level of actinorhodin production was observed. This PS produced the highest intracellular (0.54/g-cell) and extracellular (1.41/g-cell) actinorhodin, which was about ten times higher than the PNC and PC. Especially, no detectable amount of extracellular actinorhodin was produced in the PC. There were no significant differences in the amount of biomass formed among the cultures. Undecylprodigiosin production was also observed to be enhanced by pH shock (data not shown).

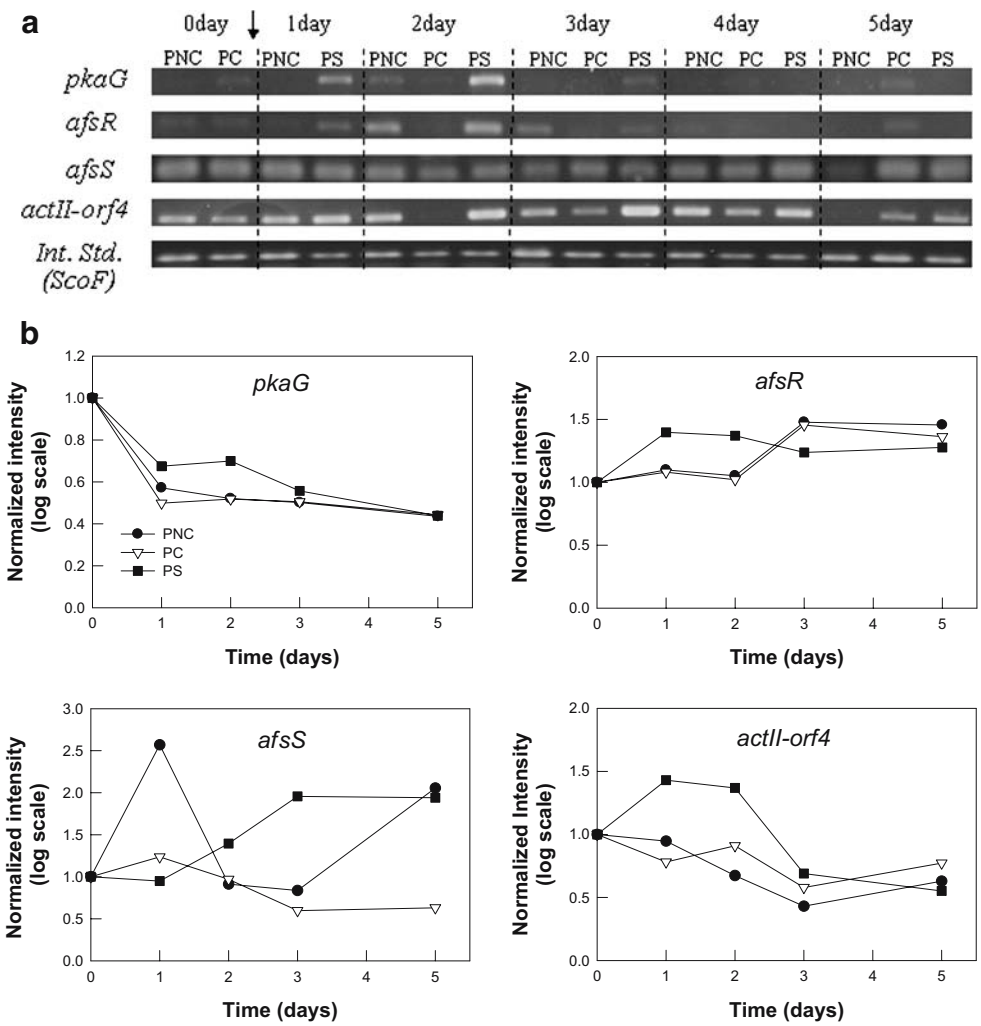


Fig. 3 Transcriptional analysis of regulators associated with actinorhodin biosynthesis. **a** Gel images from RT-PCR analysis: *PNC*, pH-noncontrolled culture; *PC*, -controlled culture; and *PS*, pH-shocked culture and **b** normalized intensities (log scale) of *pkaG*, *afsR*, *afsS*, and *actII-orf4* in DNA chip analysis

Transcriptional analysis

To investigate the effects of pH shock on the expression of signal transduction proteins and regulators related to actinorhodin production, genes involved in actinorhodin biosynthesis, and genes involved in some major metabolic pathways, transcriptional analyses were performed by RT-PCR and with DNA chips.

Result of RT-PCR analysis

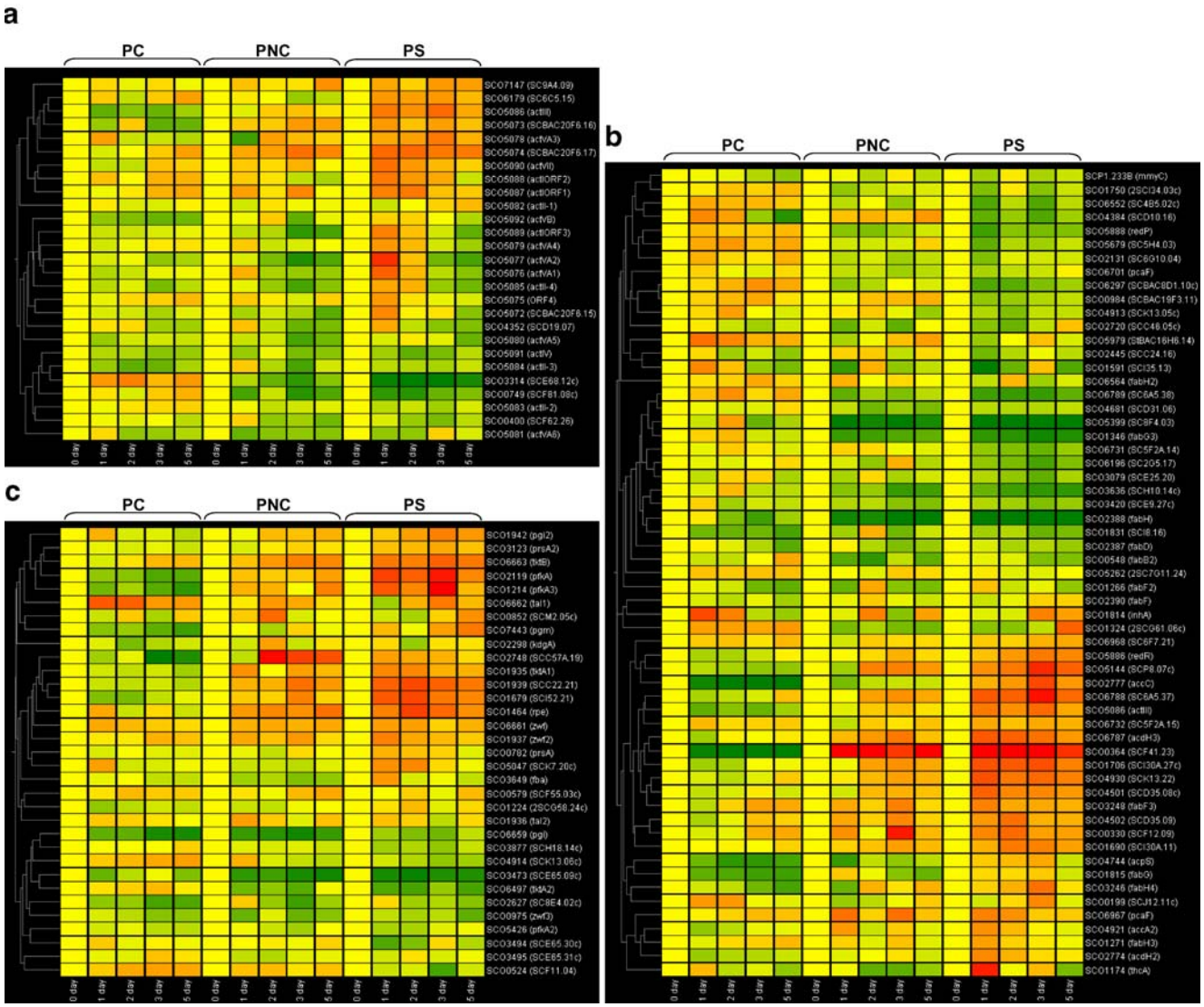
Genes encoding Ser/Thr kinases The expression of genes encoding two membrane-bound proteins of the serine/threonine kinase class, *pkaG* (SCO4487) and *afsK* (SCO4423), was analyzed. It was observed that *pkaG* expression was enhanced in the PS (Fig. 3a). In the PS, a strong band for *pkaG* appeared as early as at 1 day after pH shock. The band density reached a maximum and then rapidly decreased. The expression of *afsK* was negligible in all the cultures (data not shown).

Genes of regulators related to actinorhodin biosynthesis The effects of pH shock on the transcription of typical global and 'local' activators of regulatory protein, *afsR* (SCO4426) and sigma like protein, *afsS* (SCO4425) were observed (Fig. 3a). The *afsR* gene was maintained at a high level from 1 to 3 days after pH shock. It was highly expressed at 2 days in the PNC also, being not detected until 5 days in the PC. The *afsS* gene was maintained at a high level during the entire culture time in all the cultures without giving any noticeable evidence of pH shock effect. The pathway specific regulator gene *actII-orf4* was maintained at a high level during the whole period of culture in both the PS and the PNC. In the PC, however, its level was relatively low overall.

Results of DNA chip analysis

Regulators and gene cluster involved in actinorhodin biosynthesis Figure 3b shows the results from DNA chip analysis of four regulators involved in actinorhodin biosynthesis, *pkaG*, *afsR*, *afsS*, and *actII-orf4*. In overall, the expression levels of the regulators were observed to be upregulated in the PS earlier than the PNC and PC. The transcription of the actinorhodin biosynthetic cluster was significantly enhanced by the pH shock. Expression levels determined from the cDNA vs reference genomic DNA channel demonstrate that expression of the actinorhodin biosynthetic cluster was highly upregulated after pH shock (Fig. 4a). A pathway-specific regulator gene *actII-ORF4* (SCO5085), especially, was more highly upregulated in the PS than the PNC and PC, leading to the activation of other genes in the cluster.

Fig. 4 DNA chip analysis: *PNC*, pH-noncontrolled culture; *PC*, pH-controlled culture; and *PS*, pH-shocked culture. **a** Actinorhodin biosynthesis: genes coding hydroxylacyl-CoA dehydrogenase (SCO5072), putative oxidoreductase (SCO5073), putative dehydratase (SCO5074), integral membrane protein (*actVA1,* SCO5076), hypothetical proteins (*actVA2*, SCO5077; *actVA3*, SCO5078; *actVA4*, SCO5079), actinorhodin cluster activator protein (*actII4*, SCO5085), ketoacylreductase (*actIII*, SCO5086), actinorhodin polyketide beta-ketoacyl synthase alpha/ beta subunits (*actI1*, SCO5087; *actI2*, SCO5088), actinorhodin polyketide synthase acyl carrier protein (*actI3*, SCO5089), antinorhodin polyketide synthase bifunctional cyclase/dehydrogenase (*actVII*, SCO5090), and actinorhodin polyketide putative dimerase (*actVB*, SCO5092) were upregulated with pH shock, while genes coding putative dehydratase (SCO0749), putative dehydrogenase (SCO3314), putative actinorhodin transporter (SCO5083) were downregulated; **b** fatty acid metabolism: genes coding putative short chain oxidoreductase (SCO0330), putative enoyl-CoA hydratase (SCO0364, SCO4930), hypothetical protein (SCO1015), aldehyde dehydrogenase (*thcA*, SCO1174, SCO1706), acyl-CoA dehydrogenase (SCO1690; *acdH2*, SCO2774; *acdH3*; SCO6787), acetyl/propionyl CoA carboxylase alpha subunit (*accC*, SCO2777), 3-oxoacyl-[acyl carrier protein] synthase (*fabH4*, SCO3246; *fabF3*, SCO3248; *redR*, SCO5886), putative bifunctional protein (ketoreductase/3-oxoacyl-[acyl-carrier protein] reductase) (SCO4501), putative ketoacyl CoA thiolase (SCO4502), putative holo-[acyl-carrier protein] synthase (SCO4744), putative acyl-CoA carboxylase complex A subunit (*accA2*, SCO4921), ketoacyl reductase (SCO5086), putative acyl CoA isomerase (SCO5144), putative fatty acid oxidative multi-functional enzyme (SCO6732), and probable acetyl coA acetyltransferase (thiolase) (SCO6788) were upregulated with pH shock, while genes coding putative 3-oxoacyl-(acyl-carrier-protein) synthase (*fabB2*, SCO0548; *fabH*, SCO2388; *redP*, SCO5888; *fabH2*, SCO6564), putative 3-hydroxyacyl-CoA dehydrogenase (SCO0984; SCO6297), putative 3-ketoacyl-CoA thiolase/acetyl-CoA acetyltransferase (SCO1324), putative 3-oxoacyl-[acyl-carrier protein] reductase (*fabG3*, SCO1346), putative acyl CoA dehydrogenase (SCO1750), putative long chain fatty acid CoA ligase (SCO2131; SCO6196; SCO6552), putative thiolase (SCO3079; SCO6731), putative cytochrome P-450 hydroxylase (SCO3636), putative enoyl CoA hydratase (SCO4384, SCO5979), putative aldehyde dehydrogenase (SCO4913; SCO5679), putative dehydrogenase (SCO5262), probable acetoacetyl-coA thiolase (SCO5399), beta-ketoadipyl-CoA thiolase (*pcaF*, SCO6701), and putative fatty oxidation protein (SCO6789) were downregulated; and **c** Pentose phosphate pathway: genes coding ribose-phosphate pyrophosphokinase (*prsA*, SCO0782; *prsA2*, SCO3123), 6-phosphofructokinase (*pfkA3*, SCO1214; *pfkA*, SCO2119), putative sugar-phosphate isomerase (SCO1224, SCO2627), ribulose-phosphate 3-epimerase (*rpe*, SCO1464), putative gluconokinase (SCO1679), transketolase (*tktA1*, SCO1935; *tktB*, SCO6663), putative glucose-6-phosphate 1-dehydrogenase (*zwf2*, SCO1937; *zwf*, SCO6661), putative 6-phosphogluconolactonase (SCO1939), glucose-6-phosphate isomerase (*pgi2*, SCO1942), KHG/KDPG aldolase (*kdgA*, SCO2298), carbohydrate kinase (SCO2748), putative fructose 1,6-bisphosphate aldolase (*fba*, SCO3649), conserved hypothetical protein GlpX (SCO5047), and phosphoglucomutase (*pgm*, SCO7443) were upregulated with pH shock, while genes coding possible gluconolactonase precursor (SCO0524), putative aldolase (SCO3473; SCO3495), putative sugar kinase (SCO3494), putative 6-phosphogluconate dehydrogenase (SCO3877), putative deoxyribose-phosphate aldolase (SCO4914), and transketolase (*tktA2*, SCO6497) were downregulated



Genes associated with major metabolic pathways Genes encoding the enzymes of the glycolysis pathway, tricarboxylic acid (TCA) cycle, pentose phosphate pathway (PPP), and fatty acid metabolism were generally upregulated after pH shock or pH changes. It was noted that a collection of genes for many enzymes involved in fatty acid metabolism that were upregulated in the PC were actually downregulated in the PS, while another group of fatty acid metabolism-related genes downregulated in the PC were upregulated in the PS (Fig. 4b). A similar pattern to the PS was observed in the PNC although the upregulation/downregulation amplitudes were smaller. For example, Enoyl-CoA hydratase (SCO0364) was completely downregulated in the PC while strongly upregulated during the whole period of the PS. Among ten 3-oxoacyl-[acyl-carrier-protein] synthases catalyzing the irreversible reaction of fatty acid biosynthesis, four of them (SCO0548, SCO2388, SCO5888, and SCO6564) were downregulated with pH shock, while only two of them (SCO3246 and SCO5886) were upregulated. All of the three acyl-CoA dehydrogenases (SCO1690, SCO2774, and SCO6787) catalyzing the irreversible reaction of fatty acid degradation were upregulated. Especially, in the oxidative branch of PPP, *zwf* (SCO6661) and *zwf2* (SCO1937) encoding two different G6PDH's (glucose-6-phosphate dehydrogenases) initiating this pathway were upregulated with pH shock (Fig. 4c).

Proteomic analysis

1-DE ESI-MS/MS analysis Samples at 4 days (2 days after pH shock) were analyzed. Some proteins associated with glycolysis and TCA cycle were identified in the PNC and/or PS as listed in Table 2. They are two glyceraldehyde-3-phosphate dehydrogenases (Gap1, SCO1947 and Gap2, SCO7511), phosphoglycerate mutase (Pgm, SCO4209),

Table 2 *1-DE ESI-MS/MS* analysis results

Functional classification	Gene bank accession no.	Identity	Protein size (kDa/aa)	Protein detection		
				PNC	PC	PS
Glycolysis	SCO1947	Glyceraldehyde-3-phosphate dehydrogenase (Gap1)	36.2/336			o
	SCO4209	Phosphoglycerate mutase	28.3/253	o		o
	SCO7511	Glyceraldehyde-3-phosphate dehydrogenase (Gap2)	34.9/332	o		o
TCA cycle	SCO4827	Malate dehydrogenase	34.6/329	o		o
	SCO3127	Phosphoenolpyruvate carboxylase (Ppc)	101.3/911	o		o
Actinorhodin biosynthesis	SCO4352	Putative oxidoreductase	36.1/330	o		o
	SCO5080	Hydrolase (ActVA-ORF5)	39.8/381			o
	SCO5072	Hydroxylacyl-CoA dehydrogenase (ActVI-ORF1)	33.2/307			o
	SCO5074	Putative dehydratase (ActVI-ORF3)	23.1/214	o		o

malate dehydrogenase (SCO4827), and phosphoenolpyruvate carboxylase (Ppc, SCO3127). Also, putative oxidoreductase (SCO4352), hydrolase (ActVA-ORF5, SCO5080), hydroxylacyl-CoA dehydrogenase (ActVI-ORF1, SCO5072), and putative dehydratase (ActVI-ORF3, SCO5074) associated with actinorhodin biosynthesis were also identified in PNC and/or PS. None of these proteins were identified in the PC.

2-DE MALDI-TOF MS analysis Three runs of gel electrophoresis were performed, and the averaged results are presented in Fig. 5. For PNC, PC, and the PS, 445, 463, and 324 protein spots were detected, respectively. As a result of MALDI-TOF MS analysis, nine proteins associated with actinorhodin biosynthesis and major metabolic pathways were identified (Table 3).

Among the three glycolysis-associated proteins identified, Gap1 was strongly expressed in the PNC and the PS while its expression in the PC was very low. A putative fructose 1, 6-bisphosphate aldolase (Fba, SCO3649) and Gap2 were highly upregulated by pH shock. A putative dihydrolipoamide dehydrogenase (PdhL; SCO2180) and succinyl CoA synthetase alpha chain (SucD; SCO4809) in TCA cycle and 6-phosphogluconate dehydrogenase (SCO6658) in the oxidative branch of PPP were also upregulated by pH shock. Among the three enzymes involved in actinorhodin biosynthesis, conserved hypothetical protein of ActVA-ORF4 (SCO5079) was upregulated by pH shock. The hypothetical protein of ActVA-ORF6 (SCO5081) and ketoreductase (SCO7147) were strongly expressed in the PNC and the PS while their expression in the PC was very low.

Discussion

Enhancement of actinorhodin production and secretion

The pH shock strongly promoted both intracellular and extracellular actinorhodin production but not cell growth (Fig. 2c). In the PC, especially, the amount of extracellular actinorhodin was negligible. The PNC where spontaneous pH change was not suppressed showed a significant production of extracellular actinorhodin although much

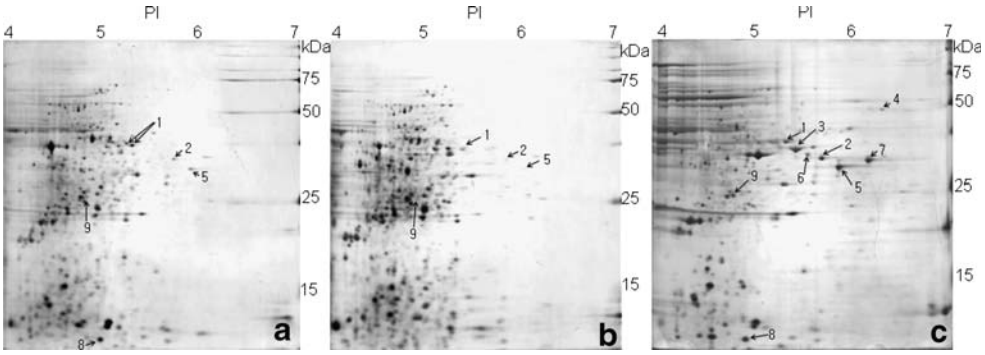


Fig. 5 Silver-stained two-dimensional gel patterns of intracellular proteins at 2 days after pH shock. **a** pH-noncontrolled culture; **b** pH-controlled culture; and **c** pH-shocked culture. Proteins with number are listed in Table 3

Table 3 *2-DE MALDI-TOFF* analysis results

Functional classification	Gene bank accession no.[a]	Identity	Protein size (kDa/aa)	Caculated pI (Expasy)	Normalized volume		
					PNC	PC	PS
Glycolysis	SCO1947 (1)	Glyceraldehyde-3-phosphate dehydrogenase (Gap1)	36.3/336	5.2	0.617	0.080	0.872
	SCO3649 (2)	Putative fructose 1,6-bisphosphate aldolase (Fba)	36.9/343	5.4	0.187	0.133	1.378
	SCO7511 (3)	Glyceraldehyde-3-phosphate dehydrogenase (Gap2)	35.0/332	5.4	–	–	1.364
TCA cycle	SCO2180 (4)	Putative dihydrolipoamide dehydrogenase	51.4/486	5.9	–	–	0.291
	SCO4809 (5)	Succinyl CoA synthetase alpha chain (SucD)	30.2/294	5.5	0.320	0.037	1.868
Oxidative branch, pentose pwy	SCO6658 (6)	6-phosphogluconate dehydrogenase	32.0/293	5.2	–	–	0.592
Type II polyketide biosynthesis Type	SCO5079 (7)	Conserved hypothetical protein (ActVA4)	32.1/294	5.7	–	–	1.377
	SCO5081 (8)	Hypothetical protein (ActVA6)	12.1/113	5.0	0.650	–	1.487
	SCO7147 (9)	Ketoreductase	26.9/255	5.9	0.151	0.056	0.59

[a] The numbers in parenthesis denote spot numbers in Fig. 5.

smaller than that of the PS. These results suggested that pH-drop-and-recovery, enforced or spontaneous, enhanced actinorhodin secretion as well as actinorhodin production.

Activation of regulators related to actinorhodin biosynthesis

The expression of four well-known regulators associated with actinorhodin biosynthesis, PkaG, AfsS, AfsR, and ActII-orf4, was observed (Fig. 3). PkaG, a serine/threonine protein kinases, phosphorylates AfsR, a global regulator (Horinouchi 2003; Lee et al. 2002; Umeyama et al. 2002). However, it has not been identified yet what kind of stimulus affects each of these two membrane-binding proteins. According to our analyses, the acidic pH shock enhanced the expression of *pkaG* probably leading to the promotion of *afsR* expression as previously reported (Sawai et al. 2004). It was reported that disruption of the chromosomal *pkaG* gene resulted in a slightly reduced actinorhodin production. It is known that AfsR regulates *afsS* and then AfsS regulates *actII-orf4* (Kim et al. 2001a, b; Lee et al. 2002). Based on this knowledge, *afsS* was expected to show a similar expression pattern to that of *afsR,* being enhanced with pH shock. Its expression was observed to be upregulated overall by pH shock or spontaneous pH change although the pattern was somewhat unexpectedly different from that of *afsR*. The expression of *actII-orf4* showed a similar pattern to that of *afsR*. This implied that there might exist another, at least, signal transduction pathway between *afsR* and *actII-orf4* bypassing *afsS*, and this pathway might have played a major role in responding to pH shock.

Overall, the pH shock was observed to trigger an early initiation of actinorhodin production by activating the regulators related to it. In flask cultures of *S. coelicolor* A3(2) M145, separately performed by our group, it was observed that actinorhodin production was initiated by pH shock even before glucose was depleted (data not shown). The regulators presented in Fig. 3 were expressed at the earliest times in the PS followed by the PNC and then by the PC.

Activation of the actinorhodin biosynthesis pathway

The pathway-specific regulator *actII-orf4* having DNA binding activity to *actVI-orf1* and *actIII-actI* intergenic region, which is necessary for the initiation of actinorhodin biosynthesis (Arias et al. 1999), was observed to be highly upregulated with pH shock as mentioned above. Such enhanced expression of this regulator is considered to have led to the activation of other genes in the actinorhodin gene cluster (Fig. 4, and Tables 2 and 3). Genes responsible for polyketide synthesis function to produce actinorhodin, ketoacylreductase (*actIII*), polyketide beta-ketozcyl synthase alpha/beta subunit (*actI-orf1* and *-orf2*), actinorhodin polyketide synthase acyl carrier protein (*actI-orf3*), and antinorhodin polyketide synthase bifunctional cyclase/dehydrogenase (*actVII*; Colombo et al. 2001; Kim et al. 1995; Sherman et al. 1992) were highly upregulated with pH shock (Fig. 4a).

Also, many other enzymes related to actinorhodin biosynthesis were observed to be upregulated by pH shock, through proteomic analysis (Tables 2 and 3). ActVI-ORF1 and ActVI-ORF3 are believed to be responsible for pyran ring closure, an intermediate step in actinorhodin biosynthesis, leading to the formation of the benzoisochromanequinone (BIQ) chromophore (Fernández-Moreno et al. 1994; Ichinose et al. 1999, 2001). Their concentrations were observed to increase during the stationary phase of *S. coelicolor* culture (Hesketh and Chater 2003). Among 6 ActVA proteins of ActVA-ORF1 ~6, ActVA-ORF4 and ActVA-ORF5 are known to play a major role in the reaction of C-6 and C-8 ring hydroxylation in the biosynthesis of aromatic polyketide antibiotics (Caballero et al. 1991).

An integral membrane protein gene (*actVA-orf1*) was observed to be strongly upregulated with pH shock through DNA chip analysis. It is known to resemble *actII-orf2* coding a putative actinorhodin transporter protein for actinorhodin secretion. These two proteins are considered to cooperate in actinorhodin export (Caballero et al. 1991; Martín et al. 2005). However, *actII-orf2* was observed to be slightly downregulated in the PS where the secretion of actinorhodin was much enhanced. These results suggested that ActVA-ORF1 might have played the major role in the export of actinorhodin under the stress condition caused by pH shock.

Activation of some major metabolic pathways by acid shock

The glycolysis pathway, TCA cycle, and PPP were, overall, enhanced in the PS compared to the PNC and PC (Fig. 4 and Tables 2 and 3). The results of DNA chip analysis showed that a number of key enzymes involved in the glycolysis pathway and TCA cycle were upregulated (data not shown). Among many key enzymes of glycolysis, two glyceraldehyde-3-phosphate dehydrogenases (Gap1 and Gap2) and fructose 1,6-bisphosphate aldolase (Fba) were observed at protein level to be upregulated in the PS. Gaps catalyze the conversion of D-glyceradehyde 3-phosphate (an important nodal compound connected to other metabolic pathways) to 3-phospho-*d*-glyceroyl phosphate. In particular, it was reported that Fba levels were higher in the stationary phase than the exponential phase in *S. coelicolor* culture (Hesketh and Chater 2003). Based on this, our result on Fba suggested that the pH shock might have triggered an early initiation of stationary or secondary metabolite production phase.

It was observed in protein level that two key enzymes involved in the conversion of pyruvate to acetyl-CoA, the putative dihydrolipoamide dehydrogenase (PdhL) and phosphoenoylpyruvate carboxylase (Ppc) were upregulated (Tables 2 and 3). The same result was obtained by DNA chip analysis. It is well known that acetyl-CoA is a precursor for polyketides biosynthesis. It was reported that amplification of the pyruvate dehydrogenase complex similar to PdhL and Ppc tended to enhance the secondary metabolites production from acetyl-CoA (Lee et al. 2006). It was reported by proteomic analysis that PdhL was induced by heat or cold shock also (Novotna et al. 2003).

The expression of many enzymes involved in fatty acid metabolism were upregulated in the PS and the PNC where pH gradient was significant but suppressed in the PC where pH gradient was relatively negligible (the lower part of Fig. 4b). Interestingly, another group of enzymes showed the opposite expression pattern (the upper part of Fig. 4b). The functional spectra (fatty acid synthesis or degradation) of these two enzyme groups were not easily distinguishable from each other. The enzymes in the first group seem to be active under a stressed condition like a severe pH change, developed by the cell itself or introduced from outside, while those in the second group under rather a normal condition. Four of ten known 3-oxoacyl-[acyl-carrier-protein] synthases catalyzing the irreversible reactions of acetyl-CoA and malonyl-CoA addition in the front part of fatty acid biosynthesis were downregulated with pH shock. All of the three acyl-CoA dehydrogenases catalyzing the irreversible reaction of fatty acid degradation were upregulated. These results implied that fatty acid biosynthesis consuming acetyl-CoA was suppressed, while fatty acid degradation was promoted in overall by pH shock. Such suppressed fatty acid biosynthesis and enhanced fatty acid degradation together were considered to have played a positive role in the promotion of actinorhodin production by providing more amount of the starting material (acetyl-CoA) and building blocks.

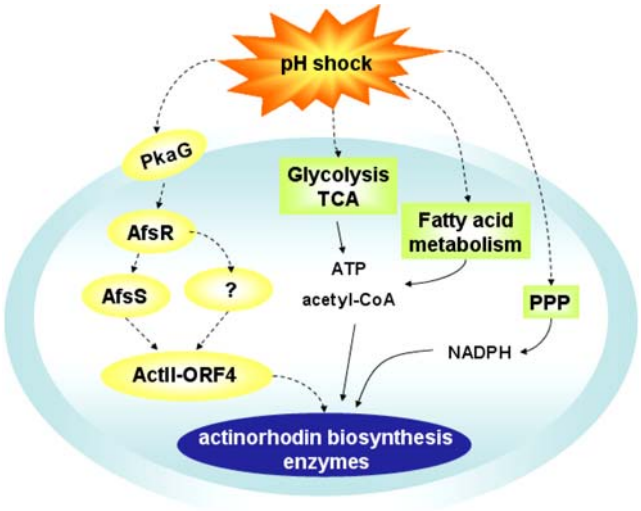


Fig. 6 Suggested schematic diagram on pH shock signal transduction to enhance actinorhodin biosynthesis in *S. coelicolor* A3(2) M145. *Solid lines* denote raw materials supply, and *dashed lines* signal transfer

According to the results of DNA chip analysis, *zwf* and *zwf2* encoding two isozymes of G6PDH were upregulated with pH shock (Fig. 4c). They are key enzymes in the oxidative branch of PPP, known to initiate PPP. In addition, many genes associated with the PPP were upregulated with pH shock. Another key enzyme, 6-phosphogluconate dehydrogenase with the same role as the protein coded by *zwf3*, was observed to be highly expressed also with pH shock through proteomic analysis (Table 3). It is known that the oxidative branch of PPP is mainly responsible for the production of reduced nicotinamide adenine dinucleotide phosphate (NADPH, the essential reducing power for biosynthesis in general (Butler et al. 2002; Pandolfi et al. 1995). Such an enhancement of PPP might have played another positive role in promoting the biosynthesis of actinorhodin.

In conclusion, major pathways of glycolysis, TCA cycle, fatty acid metabolism, and PPP were also observed to be enhanced by pH shock, most probably positively contributing to the promotion of actinorhodin biosynthesis in a concerted manner (Fig. 6).

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