Institute of Microbiology[1] and Institute of Pharmacy[2], Department of Pharmaceutical Biology, University of Greifswald, Germany

Proteomic signatures for daunomycin and adriamycin in *Bacillus subtilis*

U. Sender[1,2], J. Bandow[1], S. Engelmann[1], U. Lindequist[2], M. Hecker[1]

Received April 4, 2003, accepted May 20, 2003

Frau Prof. Dr. Ulrike Lindequist, Institute of Pharmacy, Ernst Moritz Arndt University, Friedrich-Ludwig-Jahn-Str. 17, D-17487 Greifswald
lindequi@uni-greifswald.de

Pharmazie 59: 65–70 (2004)

The influence of the two anthracyclin antibiotics daunomycin and adriamycin on the proteome of *Bacillus subtilis* was investigated. They intercalate in the double helix causing strand breaks. Both compounds induce proteins related to DNA damage and oxidative stress as indicated by the induction of some members of the PerR and the DinR-regulon. The mild induction of some members of the HrcA- and the CtsR-regulon may indicate protein oxidation as well. Furthermore, an influence on the σ^B-dependent general stress response was shown. These data show that the proteomic signature is a valuable experimental tool for a comprehensive evaluation of the action of different drugs.

1. Introduction

Because of its view on overall cell physiology, proteome analysis provides new possibilities for the target analysis of drugs. In the case of known drugs it may confirm and complete the results of biochemical or other assays, while with unknown drugs, proteome signatures give the first indications of their mode of action (Van Bogelen et al. 1999). By comparing treated cells with untreated controls, the influence of the drug on the protein synthesis pattern of cells can be followed. A prerequisite is an exact knowledge of the genome sequence of the organism which is the basis for protein identification by MALDI-TOF-MS (Park and Russel 2001). This is the case for the gram-positive bacterium *Bacillus subtilis* which has been used as a model organism for gram-positive bacteria. The complete genome sequence of this organism was published as long ago as 1997 (Kunst et al. 1997). During the last few years the proteome has been intensively investigated and proteome signatures typical of specific stimuli imposed to cells have been elaborated (Bernhardt et al. 1999; Büttner et al. 2001; Bandow et al. 2002).

Daunomycin and adriamycin are anthracyclin antibiotics. Daunomycin was isolated from *Streptomyces peuceticus* (Di Marco et al. 1964). Adriamycin is a metabolite produced by *S. peuceticus* var. *caesius* (Arcamone et al. 1969). They have antimicrobial activity against a broad spectrum of bacteria and fungi and show high cytotoxicity against eucaryotic cells. They intercalate into the DNA double helix and thereby inhibit the processes of replication, transcription and translation. DNA gyrase is probably one of the primary targets. Another assumed mechanism is the impairment of DNA by reactive oxygen species arising from the compounds (Quiles et al. 2002). Both drugs are used mainly in the treatment of tumour diseases (Acramone et al. 1969; Pearlman et al. 1985). In this study the proteome signatures induced by both drugs were analysed in order to prove whether this proteomic signature is a reasonable tool for the analysis of mode of action of drugs.

2. Investigations, results and discussion

The minimal inhibitory concentrations (MIC) of adriamycin and daunomycin were 2 µg/ml and 1.5 µg/ml, respectively. Protein synthesis rates were followed by pulse-labeling experiments with L-[^{35}S]-methionine. For these experiments a concentration of 4 times the MIC was used for both substances (Fig. 1, Fig. 2).

Crude protein extracts of cells which had been pulse-labeled at different time points after antibiotic treatment were separated on 2D-gels. To visualise proteins induced or repressed by the drug, the dual-channel imaging technique was used (Bernhardt et al. 1999). The red autoradiographs show protein synthesis after application of the substances, while the green autoradiographs represent protein synthesis under control conditions. This procedure visualises newly induced proteins by their red colour (Fig. 3).

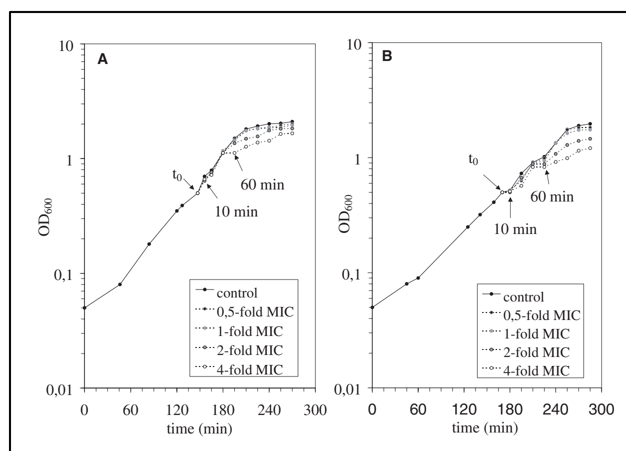

Fig. 1: *B. subtilis* 168 was grown in synthetic medium to an OD_{600} of 0.4. 4-fold MIC of adriamycin (A) and daunomycin (B) respectively were added to the cells. After 10 min and 60 min cells were pulse labeled with ^{35}S-[L]-methionine for 5 min

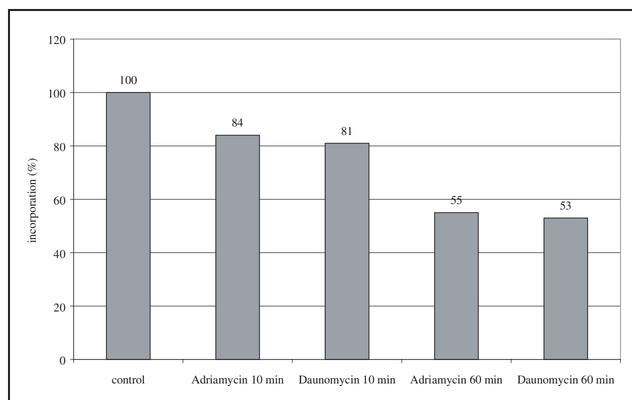

Fig. 2: Incorporation of L-[^{35}S]-methionine into 50 μg protein of *B. subtilis* 168 during 5 min was monitored at different time points after addition of 4-fold MIC antibiotics. The control sample was labeled immediately before addition of antibiotics

The Table summarizes the proteins which are induced by adriamycin or daunomycin after 10 min or 60 min treatment. A very strong induction of the DNA gyrase subunits GyrA and GyrB was found which is an agreement with literature data from eucaryotic cells (Watts et al. 2001). This induction of the gyrase subunits may be the result of the inhibition of gyrase activity by the intercalating action. GyrA and GyrB were also induced by novobiocin, a known inhibitor of DNA gyrase (Bandow et al. 2003).
Furthermore, both substances induce the SOS response in *B. subtilis* as indicated by the proteins RecA and DinB. This signature indicates DNA damage by both substances. The SOS response in *B. subtilis* is controlled by DinR, the transcriptional repressor of the system. DinR binds to the cheo-box upstream of *recA*, *dinB*, *dinC* and *dinR* (Winterling et al. 1998). RecA is believed to catalyse DinR-autocleavage, thereby derepressing the SOS-regulon in *B. subtilis* (Haijema et al., 1996). It was strongly synthesized after 10 min with an infinite induction factor (Table). RecA and DinR were also induced after treatment with mitomycin (Bandow et al. 2003).
It is well established that DNA damage is caused by oxidative stress or UV-light (Lovett et al. 1988; Setlow 1995; Gros et al. 2002). The expected oxidative stress signature induced by adriamycin and daunomycin is indicated by the strong induction of KatA and SodA and by a weaker induction of AhpC and AhpF. A similar signature was found in cells treated with hydrogen peroxide (Helmann et al. 2003; Leichert et al. 2003) or diamid (Leichert et al. 2003). The corresponding genes belong to the PerR regulon which also contains *mrgA, hemAXCDBL* or *zosA* (Fuangthong et al. 2002). The global regulator PerR, existing in distinct metalled forms is probably the target protein for oxidative stress. Therefore it cannot be ruled out at present that the oxidative stress induced by both drugs might contribute to DNA damage.
The mild induction of some members of the HrcA- or CtsR-regulon such as GroEL/GroES, GrpE or ClpC suggests that protein damage also occurs after the drug treatment but at a limited rate (Hecker et al. 1996). It is tempting to speculate that both substances induce protein oxidation such as non-native S-S-bridges or oxidation of amino acids (methionine, cysteine) which trigger the induction of chaperones or proteases (Helmann et al. 2003; Leichert et al. 2003). The increased level of Clp proteins might help to degrade irreversibly destroyed proteins. It is interesting to note that the induction of heat shock proteins in heat-treated tumor cells has been associated with adriamycin resistance (Ciocca et al. 1992).
Furthermore, MinD involved in cell division was strongly induced by both substances. MinD was induced after both

Table: Proteins induced in *B. subtilis* after treatment with adriamycin and daunomycin

Function group	Abb.	Protein	Induction factor			
			Adriamycin 10 min	Daunomycin 10 min	Adriamycin 60 min	Daunomycin 60 min
DNA	DinB	Nuclease inhibitor			∞	∞
	GyrA	DNA gyrase (subunit A)	∞	∞	∞	∞
	GyrB	DNA gyrase (subunit B)	∞	∞	∞	∞
	RecA	Multifunctional SOS repair-regulator	∞	∞		∞
Metabolism of nucleotides	Adk	Adenylate kinase	2.3	1.0	∞	∞
	Prs	Phosphoribosylpyro-phosphate synthetase	3.4		2.7	
	PurA-F[b]	Adenylosuccinate synthetase	∞	∞		∞
	PurH	Phosphoribosylamino-imidazole carboxy formyl-formyltransferase and inosine-monophosphate-cyclohydrolase			∞	∞
RNA	NusA	Transcription termination			∞	∞
	NusG	Transcription antitermination factor		1.6	2.3	1.2
	RpoB	RNA polymerase (beta subunit)	4.2	4.8	2.9	3.8
AS	Csd	Cysteine desulfurase	∞	∞		∞
	DapG	Aspartokinase I			2.0	∞
	Dat	D-alanine aminotransferase			∞	∞
	GltA	Glutamate biosynthesis			∞	∞
Protein	FusA	Elongation factor G	3.4	2.4		
	SecA	Translocase binding subunit (ATPase)	∞	∞	∞	∞
	Tsf-F[b]	Elongation factor Ts-fragment		∞	∞	∞
Glycolyse	PycA	Pyruvate carboxylase	∞	∞	∞	∞
TCC	CitB	Aconitate hydratase (aconitase)	3.2	∞	∞	∞
Oxidative stress	AhpC	Alkyl hydroperoxide reductase (small subunit)	2.9	∞	∞	∞

Table: (continued)

Function group	Abb.	Protein	Induction factor			
			Adriamycin 10 min	Daunomycin 10 min	Adriamycin 60 min	Daunomycin 60 min
	AhpF	Alkyl hydroperoxide reductase (large subunit) and NADH dehydrogenase	2.2	3.2	2.4	1.7
	SodA	Superoxide dismutase			∞	∞
	KatA	Vegetative catalase 1	∞	∞	∞	∞
	KatE	Catalase 2	∞	∞	∞	∞
	Tpx-F[b]	Thiol peroxidase-fragment			∞	
Heat stress	ClpC	Class III stress response-related ATPase	2.8	3.1	2.4	2.4
	DnaK	Class I heat-shock protein (molecular chaperone)	2.3	1.1	1.1	
	GroEL	Class I heat-shock protein (chaperonin)	3.5	2.6	3.0	5.4
	GroES	Class I heat-shock protein (chaperonin)	2.3		∞	∞
	GrpE	Heat-shock protein	2.0	∞	∞	∞
	HtpG	Class III heat-shock protein (molecular chaperone)		∞	∞	∞
General stress	Ctc	General stress protein			∞	∞
	Dps	DNA-protecting protein	3.9	2.6	1.5	
	GspA	General stress protein	∞	∞		∞
	RsbW	Negative regulation of sigma-B-dependent gene expression; phosphorylation of RsbV	3.0	∞	∞	∞
	SigB	RNA polymerase sigma-37 factor (sigma-B)	∞	∞		
Coenzyme	HemL	Glutamate-1-semialdehyde 2,1-aminotransferase	2.4	1.9	2.1	
Cell wall	GtaB	UTP-glucose-1-phosphate uridylyltransferase		3.7	2.1	2.3
Cell division	MinD	ATPase activator of MinC	∞	∞	∞	∞
Specific pathways	AckA	Acetate kinase		1.9		
Function unknown	YaaD	Similar to superoxide-inducible protein		∞	∞	∞
	YceC	Similar to tellurium resistance protein	∞	∞	∞	∞
	YceH	Similar to toxic anion resistance protein	∞	∞	∞	∞
	YceH-F[b]				1.6	
	YdcP	Similar to transposon protein			∞	∞
	YdcR	Similar to transposon protein			∞	∞
	YlaG	Similar to GTP-binding elongation factor	∞	∞	∞	∞
	YodC	Similar to nitroreductase	3.5		5.6	2.7
	YphC	Similar to unknown proteins		∞		
	YugI	Similar to polyribonucleotide nucleotidyltransferase	∞	∞	∞	
	YvyD	Unknown	∞	∞	∞	∞
	YwpJ	Similar to unknown proteins		∞		
	YwqA	Similar to SNF2 helicase			∞	
Not identified	AdA[a]	Not identified			∞	
	AdB[a]	Not identified	3.5	∞		
	AdC[a]	Not identified	∞			
	AdD[a]	Not identified	∞	∞		∞
	AdE[a]	Not identified	∞		∞	
	AdF[a]	Not identified			∞	∞
	AdG[a]	Not identified			∞	∞
	AdH[a]	Not identified			∞	∞
	AdI[a]	Not identified			∞	
	AdJ[a]	Not identified			∞	
	AdK[a]	Not identified			∞	
	AdL[a]	Not identified			∞	
	AdM[a]	Not identified			∞	
	AdN[a]	Not identified			∞	∞
	AdO[a]	Not identified			∞	∞
	AdP[a]	Not identified			∞	
	AdR[a]	Not identified			∞	∞
	AdS[a]	Not identified			∞	∞
	AdT[a]	Not identified				∞
	AdU[a]	Not identified				∞
	AdV[a]	Not identified			∞	∞
	AdW[a]	Not identified			2.1	2.5

[a] Ada to AdW are fictional names "A" stands for Adriamycin and "d" stands for Daunomycin
[b] F stands for fragment of the named protein

Fig. 3: Dual channel images of 2-D-gels produced with Delta 2D Software (Decodon) illustrate changes in protein synthesis (A) 10 min after addition of adriamycin, (B) 60 min after addition of adriamycin, (C) 10 min after addition of daunomycin and (D) 60 min after addition of daunomycin. *B. subtilis* 168 extracts before (green) and after application of substances (red). Arrows indicate identified proteins. Protein spots with an empty arrow have not been identified

10 min and 60 min by both substances with an infinite induction factor. MinD and MinC are inhibitors of regulation at medical and polar sites, and their absence leads to minicell formation (Barak et al. 1998). MinD was also induced after treatment with cerulenin and mupirocin (Bandow et al. 2003).

It is interesting to note that some members of the σ^B-dependent general stress regulon are also induced, such as GspA, Ctc, GtaB, Dps, KatE or ClpC. This is a surprising result because the general stress regulon is induced only by a severe, and not by a mild oxidative stress (Helmann et al. 2003). SigB itself and the anti-sigma factor RsbW were strongly induced after 10 min by both substances. We do not know however, why other σ^B-dependent general

stress proteins like GsiB, ClpP, TrxA and YtxH are not induced.

Finally, several proteins with unknown functions were also induced by both drugs. It is feasible to make a tentative prediction of the function of these proteins: they are probably also involved in adaptation to oxidative stress and macromolecular damage.

In conclusion these data demonstrate that the proteomic signature in *B. subtilis* found in this study is in agreement with literature data showing that both drugs induce DNA damage (Tewey et al. 1984). Furthermore, our data show that both drugs induce oxidative stress, protein stress and part of the general stress response. The question whether oxidative stress is a consequence of DNA damage or whether oxidative stress contributes to DNA stress remains to be addressed in future studies.

The main goal of the model studies was to show that the proteomic signature is an experimental approach which may be used to visualise the mechanism of action of already known substances. It turned out that the proteomic signature is a good experimental tool for evaluating the mode of action of drugs. We suggest that this proteomic signatures approach can also be used for the prediction of the mechanism of action of unknown substances. For this purpose a comprehensive knowledge of the proteomic signature for various environmental stress stimuli in a model organism (such as oxidative, acid, alkaline, heat or osmotic stress) has to be established (Hecker and Völker 2001). This physiological proteome database can be complemented by a proteomic signature library for different classes of antibiotics (Bandow et al. 2003) with reference to *B. subtilis* which might add essential new data for predicting the mode of action of drugs. Such a complex and comprehensive proteome signature library based on typical indicator proteins might be an excellent tool for the evaluation of the mode of action of drugs in general.

3. Experimental

3.1. Bacterial strains and growth conditions

B. subtilis wild-type strain 168 (*ΔtrpC2*) was cultivated under vigorous agitation at 37 °C in a synthetic medium as described previously (Stülke et al. 1993). For determination of the minimal inhibitory concentration (MIC) of the substances used in this study 1×10^5 exponentially growing cells were added to 2.5 ml of medium containing different concentrations of the substances. For growth experiments with adriamycin (Sigma-Aldrich Chemie GmbH, Munich, Germany) and daunomycin (Sigma-Aldrich Chemie GmbH, Munich, Germany) the substances were added directly to the exponentially growing cells at an optical density at 600 nm (OD_{600}) of 0.4 to a final concentration of 8 µg/ml (adriamycin) and 6 µg/ml (daunomycin), respectively.

3.2. Preparation of the cytoplasmic L-[^{35}S]methionine-labeled protein fraction

The cells were labeled with L-[^{35}S]methionine (10 µCi/ml) for 5 min at different time points after treatment with the substance, as were untreated control cells at an OD_{600} of 0.4. L-[^{35}S]-methionine incorporation was stopped by the addition of chloramphenicol (0.3 M) and an excess of unlabeled L-methionine (10 mM), as well as by transferring the culture on to ice. Cells were disrupted by ultrasonic treatment. The lysate was centrifuged (4000 g, 4 °C); the supernatant fluid was stored frozen (−20 °C).

3.3. Analytical and preparative 2D PAGE

Analytical two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) was performed using the immobilized pH gradient technique as described previousley (Völker et al. 1994). Aliquots (50 µg) of L-[^{35}S]-methionine labeled protein fractions were loaded on immobilized pH gradient strips (Amersham Pharmacia Biotech, Piscataway, N.J. USA) covering the pH range of 4 to 7. The gels were stained with silver nitrate. Afterwards the gels were dried on filter paper and exposed to Phosphor Screens (Molecular Dynamics). Phosphor Screens were detected with Phosphor imager (Molecular Dynamics). For identification of the proteins by mass spectrometry 500 µg of the protein extracts were separated by preparative 2D PAGE and the gels were stained with Sypro Ruby (Molecular Probes, Eugene, Oreg. USA).

3.4. Protein quantification

The quantification of the radioactively-labeled proteins was done using Delta 2D Software (Decodon, Greifswald, Germany). The induction factor is defined as the spot volume (substance autoradiogram) divided by the spot volume of the control autoradiogram.

3.5. Peptide mass fingerprinting

In gel-digestion with trypsin (Promega, Madison, Wis.) was performed using a peptide collection device (Otto et al. 1996). The sample preparation for protein identification by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) was performed as described previously (Bandow et al. 2002).

References

Arcamone F, Cassinelli G, Fantini G, Grein A, Orezzi P, Pol C, Spalla C (1969) Adriamycin, 14-Hydroxydaunomycin, a new antitumor antibiotic from *S. peucetius* var. *caesius*. Biotechnol Bioengineering 11: 1101–1110.

Bandow JE, Brötz H, Hecker M (2002) *B. subtilis* tolerance of moderate concentration of riampin involves the sigma B-dependent general and multiple stress resistance. J Bacteriol 184: 459–467.

Bandow JE, Brötz H, Leichert LOI, Labischinski H, Hecker M (2003) Proteomic approach to understanding antibiotic action. Antimicrob Agents Chemother 47: 948–955.

Barak I, Prepiak P, Schmeisser F (1998) MinCD proteins control the septation process during sporulation of *B. subtilis.* J Bacteriol 180: 5327–5333.

Bernhardt J, Büttner K, Scharf C, Hecker M (1999) Dual channel imaging of two-dimensional electropherograms in *B. subtilis.* Electrophoresis 20: 2225–2240.

Büttner K, Bernhardt J, Scharf C, Schmidt R, Mäder U, Eymann C, Antelmann H, Völker A, Völker U, Hecker M (2001) A comprehensive two-dimensional map of cytosolic proteins of *B. subtilis.* Electrophoresis 22: 2908–2935.

Ciocc DR, Fuqua SA, Lock-Lim S, Toft DO, Welch WJ, McGuire WL (1992) Response of human cancer cells to heat shock and chemotherapeutic drugs. Cancer Res 52: 3648–3654.

Di Marco A, Gaetani M, Orezzi P, Scarpinato BM, Silvestrini R, Soldati M, Dasidia T, Valentini L (1964) Daunomycin, a new antibiotic of the rhodomycin group. Nature 201: 706–707.

Fuangthong M, Herbig AF, Bsat N, Helmann JD (2002) Regulation of the *B. subtilis* fur and perR genes by PerR: not all members of the PerR regulon are peroxide inducible. J Bacteriol 184: 3276–3286.

Gros L, Saparbaev MK, Laval J (2002) Enzymology of the repair of free radical-induced DNA damage. Oncogene 21: 8905–8925.

Haijema BJ, van Sinderen D, Winterling, K, Kooistra J, Venema G, Hamoen LW (1996) Regulated expression of the dinR and recA genes during competence development and SOS induction in *B. subtilis*. Mol Microbiol 22: 75–85.

Hecker M, Schumann W, Völker U (1996) Heat-shock and general stress response in *B. subtilis*. Mol Microbiol 19: 417–428.

Hecker M, Völker U (2001) General stress response of *B. subtilis* and other bacteria. Adv. Microb Physiol 44: 35–91.

Helmann JD, Wu MF, Gaballa A, Kobel PA, Morshedi MM, Fawcett P, Paddon C (2003) The global transcriptional response of *B. subtilis* to peroxide stress is coordinated by three transcription factors. J Bacteriol 185: 243–253.

Kunst F, Ogasawa N, Moszer I, et al. (1997) The complete genome sequence of the gram-positive bacterium *B. subtilis.* Nature 390: 249–256.

Leichert LIO, Scharf C, Hecker M (2003) Global characterization of disulfide stress in *B. subtilis.* J Bacteriol 85: 1967–1975.

Lovett CM Jr, Love PE, Yasbin RE, Roberts JW (1988) SOS-like induction in *B. subtilis*: induction of the RecA protein analog and a damage-inducible operon by DNA damage in Rec+ and DNA repair-deficient strains. J Bacteriol 170: 1467–1474.

Otto AB, Thiede B, Müller EC, Scheler C, Wittmann-Liebhold B, Jungblut P (1996) Identification of human myocardial proteins separated by two-dimensional electrophoresis using an effective sample preparation for mass spectrometry. Electrophoresis 17: 1643–1650.

Park ZY, Russell DH (2001) Identification of individual proteins in complex protein mixtures by high-resolution, high-mass-accuracy MALDI TOF-mass spectrometry analysis of in-solution thermal denaturation/enzymatic digestion. Anal Chem 73: 2558–2564.

Pearlman LF, Simpkins H (1985) The differential effects produced by daunomycin and adriamycin on RNA polynucleotides, single stranded,

supercoiled DNA, and nucleosomes. Biochem Biophys Res Commun 131: 1033–1044.

Quiles JL, Huertas JR, Battino M, Mataix J, Ramirez-Tortosa MC (2002) Antioxidant nutrients and adriamycin toxicity. Toxicology 180 : 79–95.

Setlow P (1995) Mechanism for the prevention of damage to the DNA in spores of *Bacillus* species. Ann Rev Microbiol 49: 29–54.

Stülke J, Hanschke R, Hecker M (1993) Temporal activation of the beta-glucanase synthesis in *B. subtilis* is mediated by the GTP pool. J Gen Microbiol 139: 2041–2045.

Tewey KM, Rowe TC, Yang L, Halligan BD, Liu LF (1984) Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. Science 226: 466–468.

Van Bogelen RA, Schiller EE, Thomas JD, Neidhardt FC (1999) Diagnosis of cellular states of microbial organisms using proteomics. Electrophoresis 20: 2149–2159.

Völker U, Engelmann S, Maul B, Riethdorf S, Völker A, Schmidt R, Mach H, Hecker M (1994) Analysis of the induction of general stress proteins of *B. subtilis.* Microbiology 140: 741–752.

Watts SG, Futscher BW, Isett R, Gleason-Guzman M, Kunkel MW, and Salmon SE (2001) cDNA microarray analysis of multidrug resistance: doxorubicin selection produces multiple defects in apoptosis signaling pathway. J Pharmacol Exp Ther 299: 434–441.

Winterling KW, Chafin D, Hayes JJ, Sun J, Levine AS, Yasbin RE, Woodgate R (1998) The *B. subtilis* DinR binding site: redefinition of the consensius sequence. J Bacteriol 180: 2201–2211.