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Isoprene synthase activity parallels fluctuations of isoprene release during growth of *Bacillus subtilis*

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Abstract

Isoprene is a volatile metabolite of uncertain function in plants, animals, and bacteria. Here, we demonstrate that the isoprene-producing bacterium, *Bacillus subtilis*, contains an isoprene synthase activity that catalyzes dimethylallyl diphosphate-dependent isoprene formation. Although the enzyme was very labile, it was demonstrated in both permeabilized cells and in partially purified cell extracts. Its activity was optimal at pH 6.2, required low levels of a divalent cation, and appears distinct from chloroplast isoprene synthases. When grown in a bioreactor, *B. subtilis* cells released isoprene in three distinct phases; using permeabilized cells, it was shown that isoprene synthase activity rose and fell in parallel with each phase. These results suggest that isoprene synthesis is highly regulated in *B. subtilis* and further research in this model system may shed light on the role of isoprene formation in biological systems. © 2002 Elsevier Science (USA). All rights reserved.

Isoprene (2-methyl-1,3-butadiene) is a volatile natural product that is released by a diverse range of organisms, including plants, animals, and bacteria [1–3]. In the plant realm, it is estimated that forest isoprene emission is of the order of 500×10^6 tons C yr^{-1}, making isoprene the most abundant reactive hydrocarbon in the atmosphere [4]. The role of isoprene formation in plants is controversial and of continuing interest, because its emission from leaf chloroplasts can be a large fraction of photosynthetic carbon dioxide fixation and because its formation is regulated by light and directly linked to the recently described DOXP/MEP pathway of isoprenoid biosynthesis [1,5]. In humans, isoprene is usually the major hydrocarbon in breath [6] and its formation is of interest because of suggestions that it is derived from the cholesterol biosynthetic pathway [7]. For example, breath isoprene is lowered by treatment of individuals with HMG-CoA reductase inhibitors, such as lovastatin or lipitor [2,8], in the latter case in parallel with a decline of low density lipoproteins. These results argue that isoprene is a product or by-product of the mevalonate pathway of isoprenoid biosynthesis in humans.

The enzymatic basis for isoprene formation is best understood in plants. An isoprene synthase was purified from aspen leaves, catalyzing conversion of dimethylallyl diphosphate (DMAPP) to isoprene and pyrophosphate in a divalent cation-dependent reaction as shown in Fig. 1 [9]. Since its discovery, the enzyme has also been described in other plant species (reviewed in [1]) and localized in chloroplasts [10]. Recently, the cDNA sequence for poplar leaf isoprene synthase has been determined [11], revealing that the enzyme is highly homologous to limonene synthases which synthesize cyclic monoterpenes from geranyl diphosphate, the C10 analog of DMAPP. The mechanism of light regulation (if any) of chloroplastic isoprene synthases is unknown. Similarly, the enzymatic basis and regulation of human isoprene formation not been established.

Here, we have addressed the enzymatic basis of isoprene formation in a model bacterial system, which also shows regulated isoprene formation. Specifically, when grown in a bioreactor in glucose–tryptone medium, *Bacillus subtilis* releases isoprene in three distinct peaks that coincide with three phases of growth and differentiation: (i) glucose catabolism and acetoin secretion, (ii) acetoin reassimilation from the medium, and (iii) the early stages of sporulation [12]. The interesting pattern of isoprene release from *B. subtilis*

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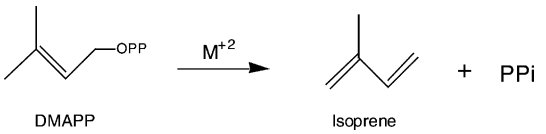

Fig. 1. The isoprene synthase reaction. The reaction is likely to proceed by divalent metal ion (M^{+2})-assisted elimination of pyrophosphate (PPi) through a carbocation intermediate, followed by proton abstraction [9].

suggests a role for the hydrocarbon, possibly as an overflow metabolite or as part of a signaling pathway, but, as in plants, this purpose has not been determined. This study describes the identification and initial characterization of an isoprene synthase in *B. subtilis*.

Materials and methods

Enzyme isolation. Isoprene synthase was extracted from the wild-type Marburg strain of *B. subtilis* 6051, which was obtained from American Type Culture Collection (Rockville, Maryland). Cells were grown in H15 medium [13] containing (per L): 10 g RNA (from torula yeast (Sigma Chemical, St. Louis, MO)), 50 g yeast extract (Difco Laboratories, Detroit, MI), 10 g casamino acids (Difco Laboratories), 1 mg RNase A (Sigma Chemical), 52.3 g 3-(*N*-morpholino)-propanesulfonic acid (MOPS)-free acid, 35 g tris-(hydroxymethyl)aminomethane (Tris-free base), and 20 g glucose. A 100 mL culture was grown aerobically for 6 h at 37 °C on a rotary shaker before being inoculated into 1 L of fresh H15 medium and grown an additional 16 h. Cells were harvested by centrifugation at 14,000*g* and washed with ice-cold water. The resulting cells were resuspended in a buffer A containing: 50 mM Tris, 2 mM DTT, 1 mM benzamidine–HCl, 1 mM PMSF, 2 mM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 2 mM Na_3VO_4, 20 mM NaF, and 10% (v/v) glycerol. The cells were lysed with lysozyme (10 mg mL^{-1}) at 37 °C for 45 min and centrifuged for 45 min at 27,000*g*. The resulting supernatant contained isoprene synthase, which was precipitated between 25% and 50% saturation with $(NH_4)_2SO_4$. The precipitate was collected by centrifugation at 27,000*g* for 10 min and resuspended in a minimal volume of buffer A. This suspension served as the enzyme sample for further experiments and was stored at −20 °C.

Partial purification of isoprene synthase. After dialysis against 1 L of buffer A, the enzyme sample was applied to a DEAE-Sephacel column (5 mL bed volume) which had been equilibrated with buffer A. The enzyme was eluted using a stepwise gradient of KCl. All steps were carried out at 4 °C. Protein concentrations were determined by the method of Bradford [14].

Assay of isoprene synthase. Production of isoprene was measured by incubating the enzyme with 1 mM DMAPP at 37 °C for 30 min, with metal ions noted in the text. The total reaction volume was 100 μL, contained in a 4.8 mL glass vial sealed with a Teflon-lined septum. Two mL of headspace was removed with a gas-tight syringe at the appropriate time and injected onto one of the two gas chromatographs, each with a 0.5 mL sample loop. The first operated isothermally at 64 °C with a flow rate of the high purity He gas at 31 cc min^{-1} [15] and contained an *n*-octane/porasil C column (Alltech Associates, Deerfield, IL). The second was built for greater sensitivity to isoprene. It ran at 100 °C with a He flow rate of 28 cc min^{-1} and was equipped with a column made of Unibeads 3S 60/80 (Alltech Associates, Deerfield, IL). Both were coupled to RGD2 reduction gas detectors (Trace Analytical, Menlo Park, CA). Isoprene production was calculated by converting GC peak area units to pmol isoprene using a calibration performed with an isoprene gas standard (Scott Specialty Gases, Longmont, CO). Controls for extracted enzyme activity were run as described with buffer A in the place of the enzyme suspension.

Bioreactor experiments. The bioreactor experiments were performed with *B. subtilis* 6051 grown in 1 L (900 mL media plus 100 mL overnight preculture) of glucose–tryptone medium (F medium) as previously described [12] at 40 °C. Growth was monitored by measuring the absorbance of aliquots at 600 nm and isoprene in the exit gas was measured using gas chromatography. Twenty-five mL aliquots of the culture were removed at various times for further analysis.

Permeabilization of cells. To assay for isoprene synthase activity, cells were permeabilized as described previously [16]. Briefly, each cell aliquot was centrifuged at 14,000*g* for 20 min and washed with ice-cold water. The resulting pellet was resuspended in permeabilization buffer (pH 6.3) at 10 mL per gram of cell wet weight, vortexed, and frozen at −20 °C for 48 h. The permeabilization buffer was identical to the assay buffer already described except that it contained 50 mM Bis–Tris instead of Tris and included 0.05% (v/v) Triton X-100. The resulting permeabilized cells were used as the enzyme sample in the assay described above. Permeabilized cells were boiled and used as a non-enzymatic control. To determine a pH optimum and metal requirements of isoprene synthase, *B. subtilis* 6051 cells were grown aerobically at 37 °C on a rotary shaker for 16 h as a 100 mL culture in Luria–Bertani (LB) medium (10 g tryptone, 5 g yeast extract, and 10 g NaCl per L) plus 5% glucose before being inoculated into 900 mL of fresh medium and grown an additional 6 h. The cells were then permeabilized as described above and assayed for isoprene synthase activity.

Results and discussion

Detection, partial purification, and characterization of an isoprene synthase from B. subtilis

An isoprene synthase activity that catalyzes the reaction shown in Fig. 1 was discovered in *B. subtilis* extracts. The activity was very labile and only reproducibly detected when suitable protectants were used in the extraction buffer. The presence of protease inhibitors in the assay buffer, particularly PMSF, allowed for the activity to be maintained for a longer period of time. Of particular interest was stabilization by phosphatase inhibitors (ammonium molybdate, sodium fluoride, sodium orthovanadate). More testing will be needed to determine whether they function to prevent dephosphorylation of the enzyme or its substrate, or have some other protective effect.

Using the above protectants, and after experimenting with various growth conditions and media, we were able to partially purify the enzyme from crude extracts of *B. subtilis* cells grown under conditions which were shown to maximize isoprene production. After ammonium sulfate precipitation and dialysis, a distinct peak of activity was eluted at 300 mM KCl from an ion-exchange chromatography column (Fig. 2). This purification step exhibited a 13-fold purification over the ammonium sulfate fraction. Further attempts at purification were hindered by loss of enzyme activity either on other columns or in storage.

Because of continuing difficulties with purification of isoprene synthase, we decided that assaying isoprene

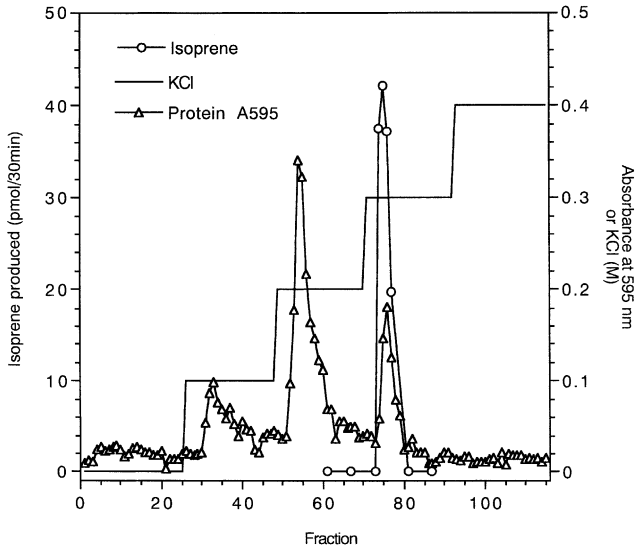

Fig. 2. Partial purification of isoprene synthase from *B. subtilis* on a DEAE-Sephacel column. As described in Materials and methods, protein was eluted using a step gradient of KCl from 0 to 400 mM. Isoprene synthase activity was determined by incubating 90 μL samples with 1 mM DMAPP for 30 min and testing for isoprene production using gas chromatography.

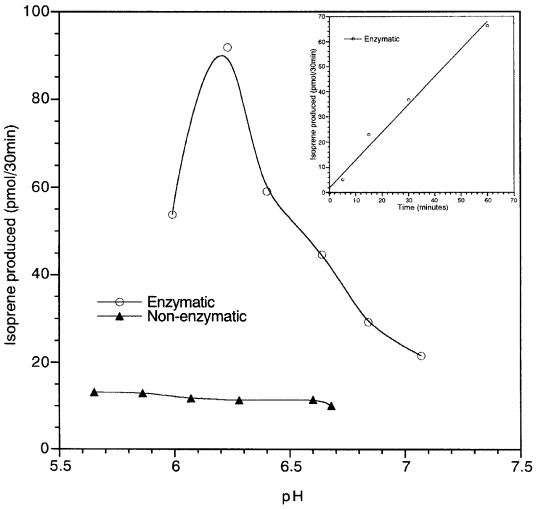

Fig. 3. pH profile for the enzymatic and non-enzymatic production of isoprene in permeabilized cells. Isoprene synthase activity was determined as described in Materials and methods, using Bis–Tris as a buffer to cover the range from pH 5.5 to 7.5. Non-enzymatic production of isoprene was determined in permeabilized cells that had been boiled to denature the enzyme. The differing pH between enzymatic and non-enzymatic samples is due to a shift in pH during boiling. Isoprene synthase activity is normalized to the OD_{600} of the permeabilized cells for each result. The inset shows representative results of the linearity of isoprene synthase activity with time in permeabilized cells. Boiled cells showed no increase in activity with time (not shown).

synthase in permeabilized *B. subtilis* cells could be a useful technique for verifying the results obtained with the extracted enzyme and for further experimentation. Permeabilization of cells can be used as a way to test an enzyme in situ [16]. Washed cells treated with Triton X-100 were shown to catalyze the DMAPP-dependent formation of isoprene. This reaction was linear with time (Fig. 3 inset). A pH profile of isoprene synthase assayed in permeabilized cells exhibits an optimum at approximately pH 6.2 (Fig. 3). A similar optimum was obtained when assaying the partially purified enzyme (not shown).

Our studies with extracted enzyme suggested that it requires metal ions but also has a sensitivity to other metal ions. Mg^{+2} and Mn^{+2} allowed for full activity at low levels (100 μM), but were inhibitory in a concentration-dependent manner; low activity was also measured in the presence of Ca^{+2}. Some metal ions abolished its activity at low concentrations (Fe^{+2}, Zn^{+2}, Cu^{+2}, Co^{+2} at 1 mM). Further, complete inhibition of activity was observed in the presence of 1 mM EDTA (data not shown). Similar results were seen when assaying isoprene synthase in permeabilized cells. As shown in Table 1, increasing amounts of Mg^{+2} and Mn^{+2} contributed to declining enzyme activity. Decreasing activity was also observed with increasing chelator concentration, with EDTA exhibiting an inhibitory effect at much lower levels than the other chelators. Of note is the increased enzyme activity in the presence of small amounts of EGTA and 1,10-phenanthroline, which is most likely due to their binding of inhibitory cations. These results, along with those from the extracted enzyme, suggest that *B. subtilis* isoprene synthase is dependent on low levels of divalent cation and is very sensitive to the concentration and identity of the metal ions in its environment.

These pH and metal ion characteristics are in striking contrast to those determined for the isoprene synthases purified from various plants. The enzymes from aspen, oak, velvet bean, and willow all exhibit pH optima between 7.5 and 8 [10,17–19], all show a dependence on

Table 1
Relative activities of isoprene synthase in permeabilized cells treated with metals and metal chelators

Treatment[b]	Activity relative to control (%)[a]		
	0.1 mM	1 mM	10 mM
Mg^{+2}	56	61	29
Mn^{+2}	85	75	16
EDTA	96	61	31
EGTA	158	129	93
1,10-Phenanthroline	121	113	80

[a] Control cells are set at 100% activity and were assayed as described in Materials and methods with no additional treatment. Each activity had background subtracted before comparisons were made to the control. The reported results are representative of multiple experiments.

[b] Metals were added as chloride salts. The metals or chelators were added at the time of the assay.

either Mg^{+2} or Mn^{+2}, but with an optimum between 10 and 15 mM Mg^{+2}, and none exhibit inhibition by increasing levels of divalent cation [10,18]. Concurrent with these differences in the prokaryotic and eukaryotic isoprene synthases, BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST/) show no homology between the recently solved isoprene synthase cDNA sequence from poplar [11] and any sequence in the genome of *B. subtilis*. Perhaps the enzyme has evolved for distinct functions in different organisms, although the possibility of similar role cannot be dismissed. Further work is needed to elucidate what this role is in both bacteria and plants.

B. subtilis isoprene synthase is a regulated enzyme

Isoprene is produced in three distinct phases when *B. subtilis* is grown in a glucose–tryptone medium [12]. To explore if these fluctuations are due to changes in enzyme activity, aliquots were removed from cells growing in a bioreactor for analysis of isoprene synthase activity. The enzyme was analyzed by assaying for its activity in permeabilized cells prepared from these aliquots. As demonstrated in Fig. 4A, isoprene synthase activity was highly correlated with the rise and fall in isoprene release that accompanies phase 1 (glucose catabolism) and phase 2 (acetoin catabolism). Similarly, its activity rose and fell significantly with phase 3 (early sporulation) isoprene release (Fig. 4B). Fig. 4C shows a separate determination of isoprene synthase activity in permeabilized cells during phase one and early phase two isoprene release. As in Fig. 4A, isoprene synthase activity was high in log phase cells, declined rapidly as glucose was depleted at the phase 1–2 transition (as indicated by pH 6.2–6.4 in the figures), and then increased rapidly as acetoin catabolism began. Differences in phase one peaks of isoprene and isoprene synthase activity (Figs. 4A and C) were frequently observed and may be due to variations in sampling times, effects of permeabilization, or other factors. Calculations of quantities of isoprene produced by cells in the bioreactor and enzymatic activity in permeabilized cells at each point give values with the same order of magnitude, suggesting that the isoprene synthase activity is responsible for the isoprene released by the cells. These results clearly show that the enzyme is regulated in some manner, and that its activity, assayed in intact cells, can change up to 4-fold in as little as 30 min.

Several factors could account for the regulation of isoprene synthase, including covalent modification, enzyme level control, or variable concentrations of inhibitors. Many enzymes in *B. subtilis* are known to be regulated by phosphorylation [20,21] and the increase in in vitro activity in the presence of phosphatase inhibitors suggests this as a possible means of regulation for isoprene synthase. *B. subtilis* also shows important

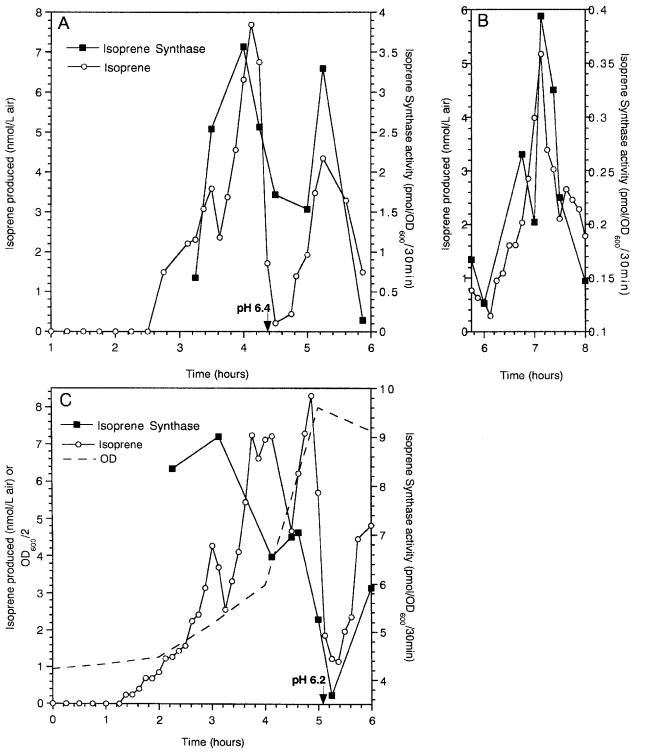

Fig. 4. Isoprene production of *B. subtilis* cells grown in a bioreactor correlates with isoprene synthase activity. (A) The first two phases of isoprene production with isoprene synthase activity in permeabilized cells withdrawn from the bioreactor. (B) The third phase of isoprene production with isoprene synthase activity assayed in permeabilized cells. (C) Cell growth, measured as optical density at 600 nm, and isoprene production during the first phase, with isoprene synthase activity in permeabilized cells. (A–C) Isoprene production and isoprene synthase activity were determined as described in Materials and methods. The isoprene synthase activity was normalized by dividing the enzymatic production of isoprene by the OD_{600} of the permeabilized cells in all cases. The pH of the medium was monitored and its lowest point, indicative of the phase 1–2 transition [12], is marked on (A) and (C). The results shown are from separate experiments, each replicated at least once.

control over proteins at the level of transcription [22]. Further, many bacteria, including *B. subtilis*, contain proteosome-like ATP-dependent protein degrading machinery, which indicates the ability of the cells to direct protein turnover [23]. Finally, because of the sensitivity of isoprene synthase to metal ions and pH, these could play some part in its regulation. Interestingly, preliminary experiments suggest that the levels of DMAPP also rise and fall during growth, as measured in aliquots removed from the bioreactor using a technique developed in our lab [24]. Isoprene is formed as part of the 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway [25] that gives rise to DMAPP, and thus these results suggest that both isoprene synthase and the supply of its substrate are regulated.

Previously, we proposed that isoprene might simply be an overflow metabolite, i.e., a secondary product that

results from overproduction of DMAPP during carbon excess [12]. *B. subtilis* can form a variety of overflow metabolites in this way [26]. However, given the results presented here we speculate that isoprene could have a more important role in *B. subtilis*, since it appears that the enzyme producing it is regulated, resulting in a periodic pattern of release from growing cells. For example, isoprene could be a intercellular signaling molecule which indicates carbon availability or central carbon assimilation rate. Further study of the *B. subtilis* isoprene synthase, including cloning and manipulation of the isoprene synthase gene, will allow us to clarify the role of this volatile hydrocarbon in bacteria and compare properties of prokaryotic and eukaryotic isoprene synthases. This research may also help to illuminate the general role of isoprene formation in plants and animals.

Acknowledgments

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References

[1] T.D. Sharkey, S.S. Yeh, Isoprene emission from plants, Annu. Rev. Plant Physiol. Plant Mol. Biol. 52 (2001) 407–436.
[2] T. Karl, P. Prazeller, D. Mayr, A. Jordan, J. Rieder, R. Fall, W. Lindinger, Human breath isoprene and its relation to blood cholesterol levels: new measurements and modeling, J. Appl. Physiol. 91 (2001) 762–770.
[3] J. Kuzma, M. Nemecek-Marshall, W.H. Pollock, R. Fall, Bacteria produce the volatile hydrocarbon isoprene, Curr. Microbiol. 30 (1995) 97–103.
[4] A. Guenther, C.N. Hewitt, D. Erickson, R. Fall, C. Geron, T. Graedel, P. Harley, L. Klinger, M. Lerdau, W.A. Mckay, T. Pierce, B. Scholes, R. Steinbrecher, R. Tallamraju, J. Taylor, P. Zimmerman, A global model of natural volatile organic compound emissions, J. Geophys. Res. 100 (1995) 8873–8892.
[5] B.A. Logan, R.K. Monson, M.J. Potosnak, Biochemistry and physiology of foliar isoprene production, Trends Plant Sci. 5 (2000) 477–481.
[6] J.D. Fenske, S.E. Paulson, Human breath emissions of VOCs, J. Air Waste Manag. Assoc. 49 (1999) 594–598.
[7] E.S. Deneris, R.A. Stein, J.F. Mead, In vitro biosynthesis of isoprene from mevalonate utilizing a rat liver cytosol fraction, Biochem. Biophys. Res. Commun. 123 (1984) 691–696.
[8] B.G. Stone, T.J. Besse, W.C. Duane, C.D. Evans, E.G. DeMaster, Effect of regulating cholesterol biosynthesis on breath isoprene excretion in men, J. Lipid Res. 28 (1993) 705–708.
[9] G.M. Silver, R. Fall, Characterization of aspen isoprene synthase, an enzyme responsible for leaf isoprene emission to the atmosphere, J. Biol. Chem. 270 (1995) 13010–13016.
[10] M.C. Wildermuth, R. Fall, Light-dependent isoprene emission—characterization of a thylakoid-bound isoprene synthase in *Salix discolor* chloroplasts, Plant Physiol. 112 (1996) 171–182.
[11] B. Miller, C. Oschinki, W. Zimmer, First isolation of an isoprene synthase gene from poplar and successful expression in *Escherichia coli*, Planta 213 (2001) 483–487.
[12] W.P. Wagner, M. Nemecek-Marshall, R. Fall, Three distinct phases of isoprene formation during growth and sporulation of *Bacillus subtilis*, J. Bacteriol. 181 (1999) 4700–4703.
[13] H.M. Duttweiler, D.S. Gross, Bacterial growth medium that significantly increases the yield of recombinant plasmid, Biotechniques 24 (1998) 438–444.
[14] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[15] J.P. Greenberg, P.R. Zimmerman, B.E. Taylor, G.M. Silver, R. Fall, Sub-parts per billion detection of isoprene using a reduction gas detector with a portable gas-chromatograph, Atmos. Environ. 27A (1993) 2689–2692.
[16] G.F. Miozzari, P. Niederberger, R. Hutter, Permeabilization of microorganisms by Triton X-100, Anal. Biochem. 90 (1978) 220–233.
[17] J. Kuzma, R. Fall, Leaf isoprene emission rate is dependent on leaf development and the level of isoprene synthase, Plant Physiol. 101 (1993) 435–440.
[18] G.M. Silver, R. Fall, Enzymatic-synthesis of isoprene from dimethylallyl diphosphate in aspen leaf extracts, Plant Physiol. 97 (1991) 1588–1591.
[19] A. Lehning, I. Zimmer, R. Steinbrecher, N. Bruggemann, J.P. Schnitzler, Isoprene synthase activity and its relation to isoprene emission in *Quercus robur* leaves, Plant Cell Environ. 22 (1999) 495–504.
[20] A. Galinier, M. Kravanja, R. Engelmann, W. Hengstenberg, M.-C. Kilhoffer, J. Deutscher, J. Haiech, New protein kinase and protein phosphate families mediate signal transduction if bacterial catabolite repression, Proc. Natl. Acad. Sci. USA 95 (1998) 1823–1828.
[21] M. Jiang, W. Shao, M. Perego, J. Hoch, Multiple histidine kinases regulate entry into stationary phase and sporulation in *Bacillus subtilis*, Mol. Microbiol. 38 (2000) 535–542.
[22] A. Klier, T. Msadek, G. Rapoport, Positive regulation in the gram-positive bacterium: *Bacillus subtilis*, Annu. Rev. Microbiol. 46 (1992) 429–459.
[23] A. Lupas, F. Zuhl, T. Tamura, S. Wolf, I. Nagy, R.D. Mot, W. Baumeister, Eubacterial proteosomes, Mol. Biol. Rep. 24 (1997) 125–131.
[24] A.J. Fisher, T.N. Rosenstiel, M.C. Shirk, R. Fall, Nonradioactive assay for cellular dimethylallyl diphosphate, Anal. Biochem. 292 (2001) 272–279.
[25] W.P. Wagner, D. Helmig, R. Fall, Isoprene biosynthesis in *Bacillus subtilis* via the methylerythritol phosphate pathway, J. Nat. Prod. 63 (2000) 37–40.
[26] M. Dauner, T. Storni, U. Sauer, *Bacillus subtilis* metabolism and energetics in carbon-limited and excess-carbon chemostat culture, J. Bacteriol. 183 (2001) 7308–7317.