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Expression of *Vitreoscilla* hemoglobin in *Gordonia amarae* enhances biosurfactant production

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Abstract The gene (*vgb*) encoding *Vitreoscilla* (bacterial) hemoglobin (VHb) was electroporated into *Gordonia amarae*, where it was stably maintained, and expressed at about 4 nmol VHb g^{-1} of cells. The maximum cell mass (OD_{600}) of *vgb*-bearing *G. amarae* was greater than that of untransformed *G. amarae* for a variety of media and aeration conditions (2.8-fold under normal aeration and 3.4-fold under limited aeration in rich medium, and 3.5-fold under normal aeration and 3.2-fold under limited aeration in mineral salts medium). The maximum level of trehalose lipid from cultures grown in rich medium plus hexadecane was also increased for the recombinant strain, by 4.0-fold in broth and 1.8-fold in cells under normal aeration and 2.1-fold in broth and 1.4-fold in cells under limited aeration. Maximum overall biosurfactant production was also increased in the engineered strain, by 1.4-fold and 2.4-fold for limited and normal aeration, respectively. The engineered strain may be an improved source for producing purified biosurfactant or an aid to microorganisms bioremediating sparingly soluble contaminants in situ.

Keywords Bioremediation · Biosurfactant production · *Gordonia amarae* · Trehalose lipid production · *Vitreoscilla* hemoglobin

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Introduction

Release of organic contaminants into the environment can be problematic because of their toxicity and their stability. Bioremediation by microorganisms, however, can often be an effective method to clean up contaminated sites [29].

Use of biologically produced surfactants as a tool in bioremediation has become a subject of increasing study, as they can dramatically increase the apparent solubility of insoluble and sparingly soluble compounds so that they can be taken up by cells and metabolized [19]. Biosurfactants are produced and released by microorganisms during growth on insoluble substrates in water [7]. They are currently used in several applications, including bioremediation, in which, unlike synthetic surfactants, they themselves are biodegradable and pose no additional pollution threat. Furthermore, most studies indicate that they are non-toxic to microorganisms and therefore are unlikely to inhibit biodegradation [2].

As with other microbial fermentations the goal in production of biosurfactants is to maximize productivity, and achieve high final concentrations [12]. In this way it is possible that solubilization and biodegradation rates of insoluble substrates can be increased by microorganisms. It has been reported that *Nocardia* species that are grown on hydrocarbons produce biosurfactants [23, 25]. In particular, the bacterium *Gordonia amarae* (previously known as *Nocardia amarae*) has been shown to produce biosurfactants that enhance removal of non-ionic organic contaminants from water [28, 36].

It has been demonstrated that expression of bacterial hemoglobin (VHb) in heterologous bacterial hosts engineered to contain the VHb gene (*vgb*) often results in enhancement of cell density, oxidative metabolism, protein and antibiotic production, and bioremediation, especially under oxygen-limiting conditions [17, 24, 35, 39, 40]. The beneficial effects of *vgb* have also been demonstrated in a variety of eukaryotes. These include

enhancement of antibiotic production in fungi [9], tissue plasminogen activator production in mammalian cells [31], and growth in plants and plant cell cultures [11, 14]. There is evidence that the beneficial effects in bacteria are the result of direct interaction of VHb with the terminal respiratory oxidase, delivering oxygen to it to enhance oxidative phosphorylation and thus the production of ATP [16, 30, 32]. In the case of bioremediation enhancement, a direct delivery of oxygen by VHb to oxygenases which degrade aromatics may occur [20, 21].

In the work reported here, *vgb* was electroporated into *G. amarae* and expressed in the resulting recombinant cells where it enhanced both growth and biosurfactant production. This is the first report in which engineering with *vgb* has been used in this way as a possible strategy to enhance biodegradation of sparingly soluble compounds. It suggests that engineering of biosurfactant-producing bacteria with *vgb* may be a useful way to extend the possible uses of *vgb* in strain improvement for bioremediation.

Materials and methods

Bacterial strains, plasmids, and media

Gordonia amarae (NRRL B-8176) was provided by the USDA National Center for Agriculture Utilization Research (Peoria, IL, USA). Plasmid pSC160 had been constructed [22] by inserting linearized pUC8:16 (which carries *vgb* on a 1.4 kb fragment from *Vitreoscilla* strain C1 [10] cloned into vector pUC8) into the *Eco*RI site of broad host range vector pKT230; pSC160 provides resistance to kanamycin and ampicillin. Routine plasmid maintenance and cloning utilized *Escherichia coli* DH5α. Selection and routine strain maintenance used plates made from LB or LB with various antibiotics (LB_{Km+Amp}, kanamycin, 40 μg ml^{-1}, ampicillin, 100 μg ml^{-1}; LB_{Sm}, streptomycin, 100 μg ml^{-1}), as appropriate.

Construction of recombinant *G. amarae* strain

Gordonia amarae chromosomal DNA was isolated using Wizard Genomic DNA purification kits (Promega). Both this DNA and pSC160 were cleaved by *Sac*I, and random *Gordonia* chromosomal DNA *Sac*I fragments were ligated into the single *Sac*I site in the plasmid. After transformation into *E. coli* DH5α, recombinant strains were selected by resistance to the antibiotics ampicillin and kanamycin and sensitivity to sulphonamide and streptomycin (as the *Sac*I site lies within the Sm^r gene in pKT230/pSC160). For routine screening, recombinant plasmids were isolated from DH5α using the method of Holmes and Quigley [15], but isolations for use for electroporation, used QIAprep Spin Miniprep Kits (Qiagen). Recombinant plasmids were confirmed by restriction analysis.

Gordonia amarae cells were made competent and transformed with recombinant plasmids via electroporation (Gene Pulser; Bio-Rad Laboratories) according to the manufacturer's protocol. After electroporation, presumptive recombinant strains were selected by resistance to kanamycin and ampicillin and sensitivity to streptomycin–sulphonamide. Wild type *G. amarae* showed natural resistance to kanamycin to some degree; therefore, kanamycin and ampicillin were used together for selection. Control electroporations using *G. amarae* cells but no DNA yielded no colonies.

Confirmation of *G. amarae* transformants

Total cell DNA was isolated from presumptive recombinant as well as untransformed *G. amarae* using Promega Wizard kits. PCR was run on these samples using primers which amplify a 0.3 kb internal portion of *vgb* [6]. Presence of VHb in presumptive *G. amarae* transformants was determined by running CO-difference spectra on whole cell extracts (late log-early stationary phase) according to the method of Dikshit and Webster [10]. Cell extracts were obtained (from presumptive transformants and untransformed cells) by suspending cells to 50 mg ml^{-1} in potassium phosphate, pH 7.2 and sonicating for 10 min, followed by centrifugation to remove cell debris. VHb levels were calculated using the extinction coefficient $E_{419-436nm} = 274$ mM^{-1} cm^{-1}. BBL Columbia CNA SB agar and MacConkey agar, as well as Gram staining, were used for further characterization of the transformant.

Growth measurements

For initial growth experiments the medium was either LB [27], or mineral salts medium [5] containing 0.5% (w/v) sodium acetate trihydrate. Single colonies of the untransformed and *vgb*-bearing *G. amarae* were inoculated into 5 ml of medium and grown overnight at 30°C and 200 rpm. For each experiment the two overnight cultures were diluted as necessary to contain an equal OD_{600}. One hundred microliter of each diluted culture (containing 0.008 to 0.062 OD_{600} units) was inoculated into the same medium (100 ml for normal aeration and 300 ml for limited aeration) in a 500 ml Erlenmeyer flask. In all cases this gave an OD_{600} at time zero of 0.000. Incubation was at 30°C at either 200 rpm (normal aeration) or 50 rpm (limited aeration). Growth was monitored by OD_{600} (samples were diluted as necessary with appropriate medium to maintain measured OD's below 0.5) and viable counts.

Although continuous oxygen monitoring was not practical for these shake flask cultures (and those described in the following section), rough adjustment of culture oxygen levels can be obtained by variation in

shaking speed and culture volume to vessel volume ratio [6, 16].

Trehalose lipid and biosurfactant measurements

For measurements of trehalose lipid and overall biosurfactant production, cells were cultured in a manner similar to that described above in LB plus 1% (v/v) hexadecane (which is known to induce biosurfactant production [8]). Inocula were prepared from overnight LB cultures as described in the previous section. For each experiment 0.8 ml containing an equal OD_{600} (0.31–0.35 OD_{600} units) was inoculated into 800 ml medium in 4 or 1.5 l shake flasks for normal and limited aeration, respectively. In all cases this gave an OD_{600} at time zero of 0.000. Homogeneity of the cultures was maintained by using vigorous agitation with a magnetic stirrer; for both normal and limited aeration the stirrer speeds were the same. Cultures were incubated at room temperature. Samples were taken periodically to measure growth (by OD_{600} as described above) and trehalose lipid concentration by the anthrone method [13]. Trehalose lipid determinations were made on both cells and cell free culture medium, which were separated from each other by centrifugation.

Samples from the cultures taken for trehalose lipid determinations were also used for measurements of surface tension. In these cases each sample was clarified by centrifugation and then filtered through a 0.45 μm filter. Surface tension of these samples was measured by the upward pull method using a Du Nuoy ring tensiometer (Fisher Model 70535) equipped with a 6-cm circumference platinum–iridium ring. Before each experiment, the ring was rinsed with distilled water and flamed to destroy any organic matter on it. Data were collected as surface tension in dynes per centimeter. The critical micelle concentration (CMC) is the concentration of biosurfactant at which the surface tension reaches its lowest value and does not decrease any further with increased biosurfactant concentration. Each sample was serially diluted with distilled water until the surface tension increased from the lowest value. The dilution factor represents the XCMC (times CMC) of the biosurfactant concentration in the culture broth [28].

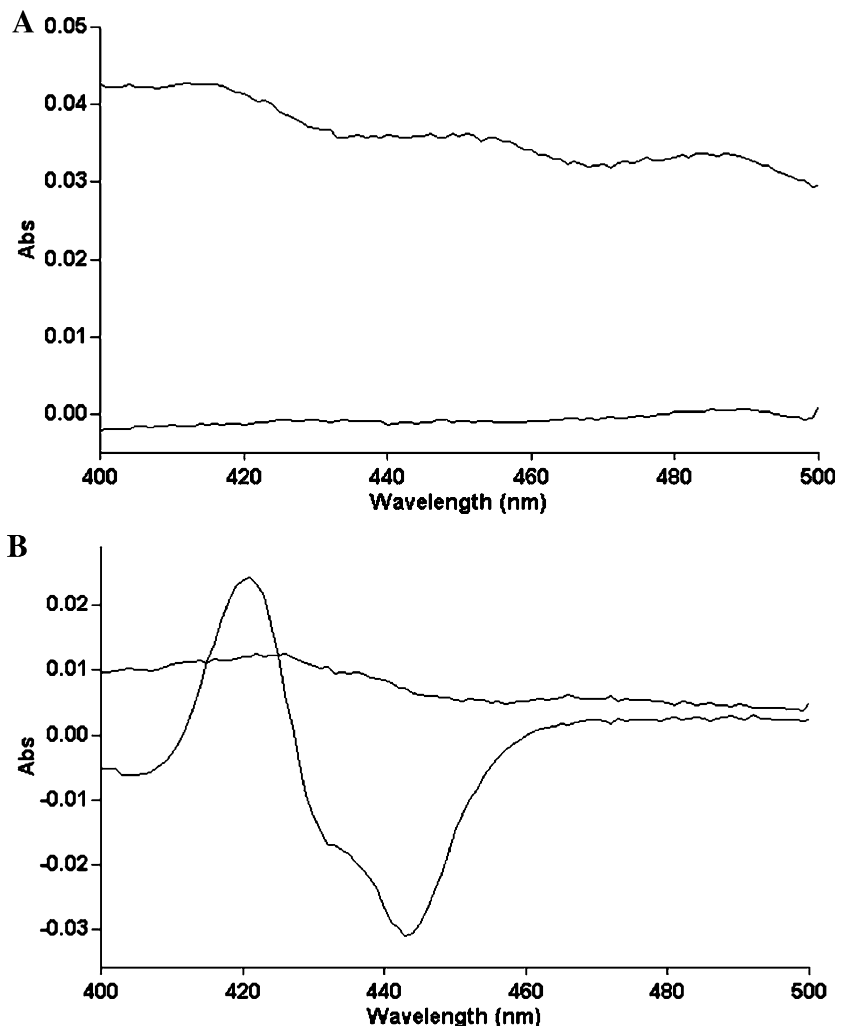

Fig. 1 CO-difference spectra of untransformed (**a**) and recombinant (**b**) *G. amarae* cell extracts. The (approximately) *horizontal line* in each panel is the baseline

Results and discussion

Construction of recombinant plasmids and selection of *G. amarae* transformants

After the ligation of random *Gordonia* chromosomal DNA *Sac*I fragments into the single *Sac*I site in plasmid pSC160 and transformation of the ligation mixture into *E. coli* DH5α, three recombinant plasmids were identified. Large amounts of these plasmids were produced and used for electroporation into *G. amarae*.

Homologous recombination between the *Gordonia* sequence within the plasmid and the corresponding sequences in the *Gordonia* chromosome presumably integrated the entire plasmid, including pSC160 and thus the *vgb* gene, into the *Gordonia* chromosome in each case (similar to what has been shown to occur in *Thermus* [18]). After 2 electroporation experiments, 11 recombinant strains were identified by resistance to kanamycin and ampicillin and sensitivity to streptomycin–sulphonamide. It is also possible that pSC160 can exist autonomously in *G. amarae*, although the likelihood for that event is unknown. Plasmid pSC160, however, is derived from plasmid RSF1010, which belongs to the Inc Q/P4 plasmid incompatibility group that can be propagated in a very extensive host range extending to Gram-positive bacteria and eukaryotes [26, 34, 37]. One of the recombinant strains (from electroporation using a recombinant plasmid with a 5.5 kb insert) was chosen for further studies.

Confirmation of *vgb*-bearing *G. amarae*

The presence of *vgb* in the recombinant *G. amarae* strain was verified by PCR using a set of primers that amplify a 0.3 kb portion of *vgb*. Untransformed *G. amarae* was used as a negative control. Only genomic DNA from the recombinant *G. amarae* produced a 0.3 kb fragment identical in size to that amplified using plasmid pSC160 as template.

VHb expression was checked using CO-difference spectra (in which VHb has a characteristic absorption maximum at 419 nm and minimum at 436 nm [10]). Cell free extracts from untransformed *G. amarae* showed neither peak nor trough, while the recombinant *G. amarae* cell free extracts showed a peak at approximately 420 nm, that was broader than that observed in *Vitreoscilla* or *E. coli* expressing VHb [10], and a trough at about 445 nm with a shoulder near 435 nm (Fig. 1). The reasons for these changes from the standard spectrum are unknown, but similar changes to the VHb CO-spectrum have also been observed in *Pseudomonas aeruginosa* [22] and *Burkholderia cepacia* [6].

The VHb level for the recombinant strain was about 4 nmole g^{-1} wet weight of cells, which is about eightfold lower than that seen in *Vitreoscilla* and about 60-fold lower than *E. coli* bearing *vgb* on a high copy number plasmid [10]. This is somewhat lower than levels which resulted in positive effects in *Pseudomonas* (8.8 nmol g^{-1}) but higher than those that were not effective in *Burkholderia* (0.8 nmol g^{-1}) [6]. It is, however, the lowest level we have found so far that is beneficial to any of the species we have transformed with the VHb gene.

Characteristics of the recombinant strain

Both transformed and untransformed *G. amarae* grew on BBL Columbia CNA SB agar (supports growth for only Gram-positive bacteria) but not MacConkey agar (supports growth for only Gram-negative bacteria). Colonies of the transformant on LB plates were about twice the diameter of those of the untransformed strain. Gram staining showed that both the untransformed *G. amarae* (approximately 90% Gram-positive and 10% Gram-negative) and the recombinant *G. amarae* (approximately 10% Gram-positive and 90% Gram-negative) were Gram-variable. Gordonia are known to be Gram-variable [1], but the reason(s) for the difference

Table 1 Growth of the untransformed (“wt”) and hemoglobin-bearing (“rec”) strains as measured by both OD_{600} and viable cell counts as described in the text

Strain, medium, aeration	OD_{600}	Time (h)
OD_{600}		
wt, min, normal aeration	0.055 (0.001)	120
rec, min, normal aeration	0.19 (0)	72
wt, min, limited aeration	0.037 (0.003)	120
rec, min, limited aeration	0.12 (0.002)	120
wt, LB, normal aeration	1.13 (0.03)	72
rec, LB, normal aeration	3.15 (0.12)	96
wt, LB, limited aeration	0.16 (0.004)	120
rec, LB, limited aeration	0.54 (0.06)	96
wt, LB + hexadecane, normal aeration	1.08 (0.12)	96
rec, LB + hexadecane, normal aeration	2.50 (0.04)	120
wt, LB + hexadecane, limited aeration	0.80 (0.14)	144
rec, LB + hexadecane, limited aeration	1.45 (0.45)	144
Viable cell counts		
wt, min, normal aeration	2.63E + 08 (2.30E + 07)	72
rec, min, normal aeration	2.54E + 09 (7.21E + 07)	72
wt, min, limited aeration	8.00E + 07 (2.07E + 06)	72
rec, min, limited aeration	1.10E + 09 (8.02E + 07)	96
wt, LB, normal aeration	5.00E + 09 (1.97E + 07)	48
rec, LB, normal aeration	3.00E + 10 (1.91E + 09)	48
wt, LB, limited aeration	5.11E + 08 (8.00E + 05)	24
rec, LB, limited aeration	1.70E + 09 (7.21E + 07)	96

Data are maximum values for growth under both normal and limited aeration, and in LB, LB plus 1% v/v hexadecane, and mineral salts (“min”) media. Values are averages of three replicates (standard deviations in parentheses). Data were collected for 144 h for the experiments using LB plus hexadecane and 120 h for all other experiments; data points were taken at 0 h (inoculation) and at 4–24 h intervals thereafter; values in the rightmost column are times at which each maximum occurred. Experiments utilizing LB plus hexadecane measured only OD_{600} (and not viable counts)

we see between transformed and untransformed strains in this property is not known. Gram variability has been shown to be related to the growth stage for a number of bacteria [3], which indicates that changes in the physiological state of cells could be its cause. The presence of *Vitreoscilla* hemoglobin (VHb) in *E. coli* cells has been shown to result in significant changes in the expression of hundreds of genes compared to cells which were not transformed with VHb [33]. If similar changes in gene expression occur in *Gordonia*, they might also lead to significant physiological changes which, in turn, affect Gram staining.

Despite its difference from the untransformed strain in Gram staining and colony characteristics, the identity of the transformed strain as *G. amarae* is supported by the following: (a) control electroporations using our *G. amarae* cultures and no DNA never gave any colonies, lessening the probablility that some contaminant was present in the electroporated samples; (b) the transformants had precisely the drug resistance/sensitivity expected, which would be unlikely to occur from a random contaminant; (c) both transformant and untransformed cells grew on CNA SB agar, but neither grew on MacConkey agar; and (d) both transformant and untransformed cells produced extracellular biosurfactant and trehalose lipid.

The transformed strain was stable as indicated by its retention of both the antibiotic resistance provided by the vector pSC160 (which will remain with *vgb* whether on the plasmid or integrated into the host chromosome) and colony morphology upon repeated transfer of the recombinant strain over many months.

Growth comparisons

Growth of untransformed and *vgb*-bearing *G. amarae* was compared in three different media for both normal and limited aeration (Table 1). In both LB and mineral salts medium plus sodium acetate and at both aerations the presence of *vgb*/VHb provided a substantial advantage regarding both maximum OD_{600} and maximum viable cells. The advantages regarding OD_{600} ranged from 2.8X to 3.5X and were similar at both aerations. The advantages regarding viable cells ranged from 3.3X to 14X and were greater in mineral salts medium. When cells were grown in LB plus hexadecane the advantages in maximum OD_{600} were 1.8X and 2.3X for the VHb producing cells at limited and normal aerations, respectively. These figures are within the ranges seen in previous work. In some cases the presence of VHb in recombinant bacteria has resulted in no growth

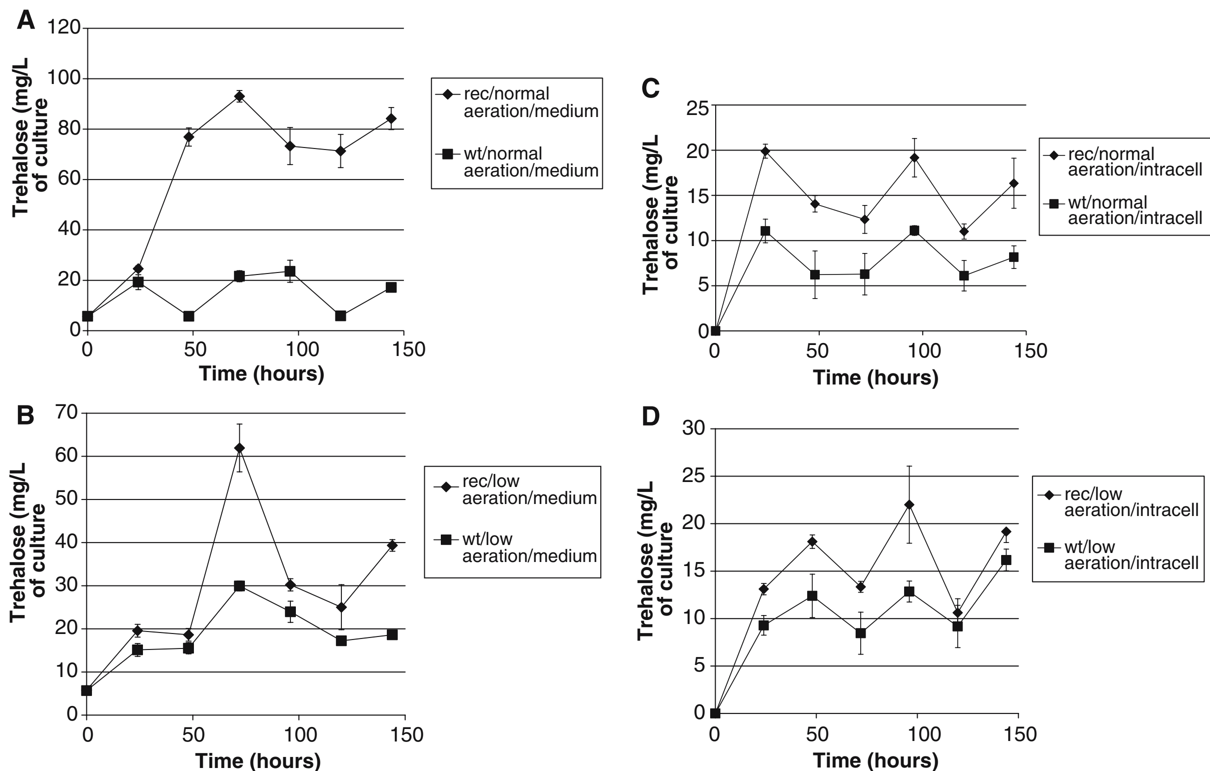

Fig. 2 Intra- and extracellular (*medium*) trehalose lipid concentrations (expressed as milligram trehalose per liter of culture) for the recombinant (*rec*) and the non-transformed (*wt*) strains grown under both normal and low aeration. **a** Medium concentrations for normal aeration; **b** medium concentrations for low aeration; **c** intracellular concentrations for normal aeration; **d** intracellular concentrations for low aeration. Times indicate hours after inoculation that samples were taken. Growth data for the cultures are the last four entries in Table 1, OD_{600}; late log phase was about 72 h for the recombinant strain under low aeration and about 48 h otherwise. Values are averages of three individual measurements. *Error bars* indicate standard deviations

advantage or even a slight growth disadvantage compared to VHb-free cells [4, 22], and sometimes to advantages as high as 2–3X on a dry cell mass basis [38] and as high as about 40X on a viable cell basis [39].

Trehalose lipid and overall biosurfactant determinations

Growth in LB plus hexadecane was also used to measure trehalose lipid production. This compound is a component of *G. amarae* produced biosurfactant, although its percentage of total biosurfactant is unknown. Biosurfactants include a variety of compounds, among which glycolipids are the major fraction [12]. As trehalose lipid is one of these glycolipids, its measurement is a useful indicator of biosurfactant production. Our measurement of overall biosurfactant levels (discussed below) supplemented the trehalose data to provide a more complete picture of biosurfactant levels. Hexadecane was included in the medium, as biosurfactant production is significantly greater when a hydrophobic carbon source is added to the growth medium [28]. The details of the genetics of this hexadecane effect, however, are not known.

The presence of VHb enhanced trehalose lipid production in the recombinant strain in both the broth and in cells (Fig. 2). Under normal aeration, the recombinant strain improved the maximum trehalose lipid level in the medium and cells by 4.0X and 1.8X, respectively. Under limited aeration, the same advantages of the recombinant strain were 2.1X and 1.4X, respectively. These differences in maximum biosurfactant levels are significant because biosurfactant would be harvested in batch experiments at these peaks. The levels of both intra- and extracellular (i.e., medium) trehalose lipid were determined for sake of completeness. The extracellular levels are perhaps most important, however, because of the relative ease of harvesting trehalose lipid from the medium.

The levels of trehalose lipid measured for *G. amarae* by Pagilla et al. [28] were similar to those found in this study both in order of magnitude and in the cyclical nature of maxima and minima as a function of time in culture. This cyclical pattern is thought to be due to repeated periods of biosurfactant production, consequent solubilization of hexadecane, uptake of hexadecane and biosurfactant, and biodegradation of biosurfactant.

Another measure of biosurfactant production during growth is surface tension of the culture medium, which decreases with increased biosurfactant production, and after reaching a threshold, does not change further. As mentioned above, this threshold biosurfactant concentration is called the CMC. This technique is commonly used to measure the extent of biosurfactant production and is represented as XCMC [28]. For growth at both normal and limited aeration there was a greater production of biosurfactant (by this measurement) for the *vgb*-bearing strain as compared to its untransformed counterpart, and these differences paralleled roughly the differences seen in trehalose lipid production between the two strains (Fig. 3). As was the case for trehalose lipid, the difference between *vgb*-bearing and untransformed strains was greater at normal than at limited aeration.

That the time courses of trehalose lipid measurements and biosurfactant levels determined by the surface tension assay are not identical is perhaps not surprising, since the surface tension data are a measure of total biosurfactant, of which trehalose lipid is only one component.

The presence of VHb is usually, although not always, most effective regarding its positive effects when cells are grown in limited aeration conditions [35]. In the case of *G. amarae*, the positive effects (growth, trehalose lipid production, overall biosurfactant production) are similar or greater at normal versus limited aeration. The reasons for this are not known, but in *Vitreoscilla*, *E. coli*, and perhaps other bacteria, VHb levels increase under hypoxic conditions (thus, presumably leading to a greater relative advantage of VHb-producing cells when aeration is limited), and this may not occur in

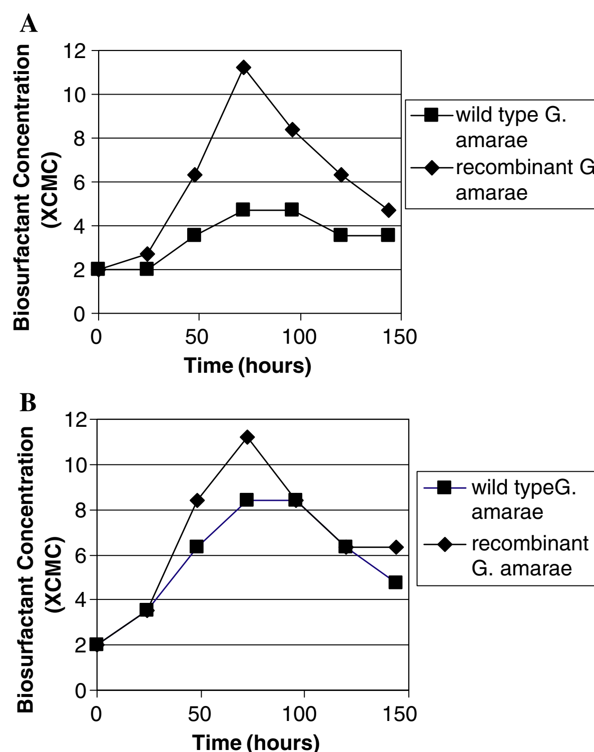

Fig. 3 Biosurfactant concentration [at normal (**a**) and limited (**b**) aeration] in cell free culture medium (measured in terms of XCMC) versus growth time. For each time point, data from identical dilution series of medium from three replicate cultures were averaged and used to construct a surface tension versus log dilution plot. From each plot, one of the data points in **a** and **b** was calculated, using the method referenced in the text. Times indicate hours after inoculation that samples were taken. Growth data for the cultures are the last four entries in Table 1, OD_{600}; late log phase was about 72 h for the recombinant strain under low aeration and about 48 h otherwise

G. amarae. It is also possible that the uptake of dissolved oxygen (DO) may be very high when hexadecane is present in the growth medium as an additional carbon source, and limited aeration conditions may not be sufficient to provide sufficient DO, even with VHb. In fact, Pagilla et al. [28] used pure oxygen instead of air to supply DO when 1% hexadecane was used for biosurfactant production with this species.

In summary, then, genetic engineering of biosurfactant producing strains with *vgb* may be an effective method to increase biosurfactant production. This approach may be useful in both in situ applications, where inoculation of an engineered bacterial strain into a site contaminated with a sparingly soluble chemical can continuously produce biosurfactant to enhance metabolism of this compound by itself or another species, or for enhanced production of biosurfactant for purification and utilization in that form to replace synthetic surfactants.

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