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ORIGINAL PAPER

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# Bioengineered emulsans from *Acinetobacter calcoaceticus* RAG-1 transposon mutants

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**Abstract** Transposon mutants of *Acinetobacter calcoaceticus* strain RAG-1 were studied in an effort to control fatty acid (FA) substitution patterns of emulsan, a bioemulsifier secreted by the organism. The disrupted genes, involved in the biosynthetic pathways of biotin, histidine, cysteine or purines, influenced the level and types of FAs incorporated into emulsan. The structural variants of emulsan generated by the transposon mutants were characterized for yield, FA content, molecular weight, and emulsification behavior when grown on a series of FAs of different chain lengths from C11 to C18. Yields of emulsan from the transposon mutants were found to be lower than the parent strain and depended on the type of FA used to supplement the growth medium. Mutants 13D (His–) and 52D (Cys–) grown on LB plus C16 or C14, respectively, exhibited enhanced emulsifying activity compared to *A. calcoaceticus* RAG-1. The presence and composition of long chain FAs on the polysaccharide backbone influenced emulsification behavior: particularly a high mole percentage of C16 (48%) and C18 (42%). The results provide important insight into the bioengineering of bioemulsifier-producing microorganisms and provide a path towards highly tailored novel amphipathic structures to utilize as biodegradable in environmental, biomedical, and personal care applications.

## Introduction

*Acinetobacter calcoaceticus* RAG-1 (ATCC 31012) can metabolize a variety of carbon sources including crude oils, long chain hydrocarbons, alcohols, fatty acids (FAs), and triglycerides. During growth, the bacterium secretes an anionic lipoheteropolysaccharide known as emulsan (Zuckerberg et al. 1979). Saturated and monounsaturated FAs ranging from C10 to C18 are linked to the polysaccharide backbone by *O*- and *N*-acyl bonds and constitute between 5 and 23% (w/w) of the polymer (Zhang et al. 1997). The combination of a hydrophilic anionic sugar main chain with hydrophobic FA side chain provides the amphipathic properties to the polymer. These chemical characteristics make emulsan a useful biological emulsifier; having been shown to stabilize 70% oil/water emulsions (Zosim et al. 1982).

We have recently demonstrated that the chemistry of the polymer can be significantly altered through the use of microbial physiological approaches (Gorvenko et al. 1997, 1999; Zhang et al. 1997, 1999). The bacterium was able to incorporate exogenous FAs into the emulsan structure, resulting in alterations in chemistry and solution properties of the family of emulsans biosynthesized. Variations in FA side chain distributions (e.g., chain length, degree of incorporation) and chemistry (e.g., saturation and hydroxylation) were reported. Based on these studies, we have now explored genetic modifications of *A. calcoaceticus* RAG-1 in an effort to further understand and control FA substitution patterns on the polysaccharide backbone.

The objective of the present study was to characterize the chemical modifications of emulsans that were generated through genetic alterations of the parent strain in an attempt to further expand the range of structural variants of emulsan that can be biosynthesized. Transposon mutants of *A. calcoaceticus* RAG-1 were screened and the emulsans produced were isolated and characterized for structural features and emulsification behavior. We report on the types of FAs, molecular weights, and change in emulsification behavior when the parent strain and the transposon mutants were grown on a series of FAs. The genomic DNA surrounding the site of transposon insertion in four mutants with altered emulsan profiles was also cloned and sequenced. In doing so, we have identified genes that appear to be indirectly involved in the biosynthesis of emulsan that influence the FAs incorporated into the polymer.

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## Materials and methods

### Strains, culture conditions, and emulsan purification

*A. calcoaceticus* RAG-1 (ATCC 31012) was obtained from the American Type Culture Collection. Unless otherwise indicated, chemicals and media components were purchased from Fisher Scientific and Sigma. RAG-1 transposon mutants 13D (histidine–), 52D (cysteine–), 62C (adenine–), and VRBS1 (biotin–) were generated using the mini-Tn*10*PttKm transposon (Leahy et al. 1993). Growth of the mutants was tested on defined mineral medium plates (containing per liter: $K_2HPO_4 \cdot 3H_2O$, 22.2 g; $H_2PO_4 \cdot 7.26$ g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $(NH_4)_2SO_4$, 4 g; either ethanol, 15 ml, or palmitic acid, 10 g, as a carbon source; and agar, 15 g, supplemented with cysteine (50 mg/l), biotin (1 mg/l), histidine (20 mg/l), or adenine (20 mg/l).

The parent strain and the transposon mutants of *A. calcoaceticus* RAG-1 were grown on Luria Bertani (LB) broth with undecanoic acid, myristic acid, palmitic acid, and stearic acid FAs (1% w/v) as carbon sources in 500 ml baffled flasks containing 100 ml medium and incubated at 30°C in an orbital shaker (250 rpm) for 6 days.

The methods for purification of emulsan were described earlier (Gorvenko et al. 1997). Cell cultures were harvested by centrifugation (30 min, 10,000 rpm) and the polymer was precipitated by the addition of ammonium sulfate to approximately 40% saturation while the solution was maintained at 4°C. The precipitated product was isolated by centrifugation, desalted by dialysis (Spectrum, MW cut-off 6,000–8,000 Da), and lyophilized. Residual aliphatic impurities were removed by Soxhlet extraction with ether. Associated proteins were removed from the polymer by hot phenol extraction (Zuckerberg et al. 1979).

### FA analysis

The FAs covalently coupled to the polysaccharide backbone were removed from the polymer after the hot phenol treatment and analyzed by GC-MS to establish composition and the degree of substitution (nmol of FA/mg of emulsan) using previously published methods (Belsky et al. 1979; Gorvenko et al. 1997). Standards (purchased from Aldrich) were used to confirm mass spectra of the FAs and to obtain relative response factors for quantitative analyses (Belsky et al. 1979; Zhang et al. 1997). The product obtained was dissolved in a mixture of 2 ml of chloroform containing 200 µl of 1.5 mg/ml tetradecane as an internal standard, filtered through a 0.45 µm syringe filter, dried using an argon stream and dissolved in 50 µl of chloroform. The FA composition was determined by gas chromatography (Hewlett Packard 5980) coupled to a mass selective detector (HP 5988 series). The column was an SE54 capillary column (5% diphenyl, 95% dimethyl polysiloxane, 30 m, i.d., 0.32 mm). The conditions were: 1 min isotherm at 140°C followed by 5°C/min ramp to 290°C, and then 5 min isothermal at 290°C. A splitless injector (290°C) and GC interface at 240°C were used for the analysis. Mass spectra were operated in electron ionization positive mode. Analysis of the samples was performed using a W search mass spectral program (version 1/10/99 C). Mass spectra of peaks were compared with those in the instrument data bank as well as with mass spectra collected for methyl esters of the FA standards.

### Molecular weight

Molecular weight of the polysaccharide backbone of the emulsan was determined after removal of FAs. Samples were analyzed by gel permeation chromatography (GPC) using Waters Millenium 32 software. The GPC consisted of a Waters 2690 separations module and Waters 410 differential refractrometer with a Waters Ultrahydrogel column packed with hydroxylated polymethacrylate (6–13 µm, 7.8×300 mm). Samples of 50 µl (from 2 mg of polysaccharide backbone dissolved in 2 ml of 0.35% LiBr) were injected in each run. The mobile phase was water containing 0.35% LiBr at a flow rate of 0.5 ml/min. Pullulan standards, 5,900 to 788,000 Da, were used for calibration (Polymer Laboratory, Amherst, Mass.).

### Emulsification assay

Emulsification activity was determined by a modification of a published procedure (Rosenberg et al. 1979). Mixtures containing 2 mg of the emulsan analog, 0.2 ml of a substrate (hexadecane, tetradecane or dodecane), and 15 ml of 20 mM Tris/10 mM $MgSO_4$ (pH 7.2) were introduced into 100 ml baffled flasks and incubated at 30°C with shaking at 150 rpm for 1 h. Turbidity of the assay mixtures was assayed with a Klett-Summerson colorimeter (green filter) after allowing the mixture to settle for 10 min. Emulsification activity was expressed as turbidity in Klett units (Zhang et al. 1999).

### DNA sequence analysis

Genomic DNA purifications and Southern blotting were performed by standard methods (Ausubel et al. 1995). Partially *Eco*RI-digested DNA from mutants 52D and VRBS1 was cloned into cosmid pcosRW2 (Wenzel and Herrmann 1988), while DNA from mutants 13D and 62C was digested with *Xba*I and *Eco*RI and ligated into pBluescriptII SK+ (Stratagene). *Escherichia coli* clones carrying the transposable element were selected on medium containing kanamycin (30 µg/ml) and plasmid DNA was purified using Qiagen kits. DNA flanking the transposon insertion sites was sequenced using automated cycle sequencing (Prism Model 377 and BigDye Terminator kit; ABI) at the Tufts Core Facility by primer walking beginning with primers internal to the transposon (APH2, 5′-GGACGGCGGCTTTGTTG-3′; and BAR5, 5′-CCTCGGTGGCACGGCGGATGT-3′). Sequences were compared against public databases using BLASTX (www.ncbi.nlm.nih.gov).

### Statistics

Data presented in Tables 2, 3 and 4, and Fig. 1 were analyzed by students T-test to compare differences between *A. calcoaceticus* RAG-1 and the mutants at $P < 0.05$.

## Results

### Selection of mutants and production of emulsan

The present study was focused on emulsan produced by *A. calcoaceticus* RAG-1 and the transposon mutants. The goal was to explore modifications in FA metabolism (e.g., the inability to utilize FAs as carbon sources or the inability to synthesize FAs) as a basis for selection of mutants to examine change in structural features of the emulsan. Three nutritional auxotrophs (13D, 52D, and 62C) capable of growth on LB but not on minimal medium, and a FA biosynthesis-deficient mutant (VRBS1), capable ofgrowth on minimal medium with FAs, but not ethanol as sole carbon source, were chosen for further characterization.

### Characterization of disrupted genes

Single insertion sites in the *A. calcoaceticus* genome were confirmed for each of the selected mutants based

**Table 1** Genetic characterization of *Acinetobacter calcoaceticus* RAG-1 transposon mutants

| Mutant (accession number) | Phenotype | Nearest homolog of disrupted ORF / amino acid identity[a] | Disrupted pathway |
|---|---|---|---|
| VRBS1 (AF239257) | Growth on minimal (M) medium + C16, no growth on M medium + ethanol | *Escherichia coli* BioB (biotin synthase) / 63% | Fatty acid (FA) biosynthesis |
| 13D (AY035546) | No growth on M medium | *Pseudomonas aeruginosa* HisA (phosphoribosylformimino-5-aminoimidazole carboxamide isomerase) / 77% | Histidine biosynthesis |
| 52D (AF239256) | No growth on M medium | *P. aeruginosa* CysI (sulfite reductase hemoprotein subunit) / 65% | Cysteine biosynthesis |
| 62C (AY035547) | No growth on M medium | *Deinococcus radiodurans* PurK (phosphoribosylaminoimadazole carboxylase ATPase subunit) / 55% | Purine biosynthesis |

[a] Determined by BLASTX of DNA sequence

on Southern hybridizations using the transposon as a probe (data not shown). The site of transposon insertion was determined for each mutant by cloning the genomic DNA fragment conferring kanamycin resistance and sequencing outward using primers complimentary to sites within the Tn*10*PttKm cassette. The results of homology searches with the resulting sequences are shown in Table 1. Supplementation of minimal medium plus ethanol with biotin, cysteine, histidine, and adenine permitted growth of VRBS1, 52D, 13D, and 62C, respectively, verifying the roles of the disrupted genes. Growth curves of the parent strain and the four transposon mutants after 6 days revealed similar cell densities of $2.7\times10^7$–$3.4\times10^7$/ml.

Yields of emulsan were determined at various steps of purification as described in Table 2. A number of differences were observed when the yields of emulsan from mutants were compared with that of *A. calcoaceticus* RAG-1. Mutant 52D (grown on LB+C14) generated 72 mg of ether-extracted emulsan as compared to 107 mg from RAG-1. Mutants 62C, 13D and VRBS1 (grown on LB+C18) generated a maximum of 30, 17 and 13 mg of apo-emulsan (after hot phenol treatment), respectively, compared to 59 mg from RAG-1. Measurements of yield based only on the polysaccharide backbone of emulsan ranged from 1 to 5 mg from the mutants, except for 13D and 62C (grown on C18) where 11 mg of polysaccharide was obtained, compared to 13 mg in *A. calcoaceticus* RAG-1. In the parent strain and the mutants grown on LB alone, the yields of emulsan were very low.

## FA analysis

FA profiles determined for the emulsans produced by the parent strain and the four transposon mutants are shown in Fig. 1. In the case of *A. calcoaceticus* RAG-1 grown on LB alone, the total FA content per milligram of emulsan was 15 nmol. This finding contrasts significantly with emulsan generated by *A. calcoaceticus* RAG-1 grown on defined media with ethanol where 443 nmol FA/mg emulsan was present. The FA content was lower in all cases for the mutants (Fig. 1). An increase in the FA content of emulsan was only observed when *A. calcoaceticus* RAG-1 was grown on LB+C11 (269 nmol/mg), LB+C14 (604 nmol/mg), and LB+C16 (680 nmol/mg) (Fig. 1). In the case of *A. calcoaceticus* RAG-1 grown on LB+C11, LB+C14, and in both cases of LB+C16 and LB+C18, a large mole percentage of C18, C14, and C12 (i.e., 33%, 52%, and 42% and 33%, respectively), was observed (Table 3). Significant changes in FA incorporation profiles were observed when the mutant 13D was characterized. Specifically, when grown on LB+C11 and LB+C14, 33 nmol of C14 and 76 nmol of C16 were present, respectively, on the polysaccharide backbone (Table 3), suggesting direct incorporation of this exogenous FA. However, only a total of 94 nmol and 157 nmol FA/mg emulsan was present, respectively, (Fig. 1) suggesting that FA metabolism is severely altered and the small amount appended to the polysaccharide backbone was derived directly from the

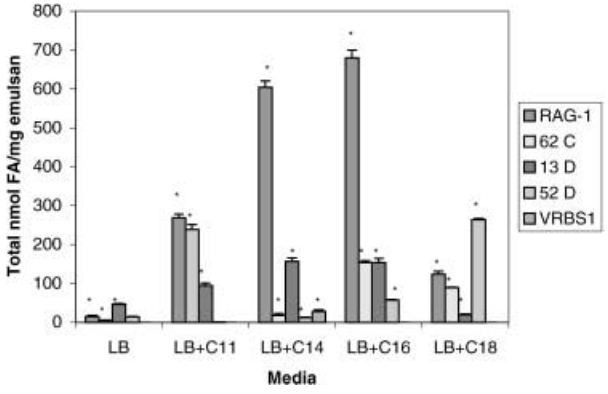


**Fig. 1** Total fatty acid (FA) content of emulsan analogs. *Error bars* Standard deviation of the means, * $P$ <0.05 compared to *Acinetobacter calcoaceticus* RAG-1

**Table 2** Emulsan yields after growth on Luria Bertani (LB) broth plus FAs at different purification steps

| Strain | Carbon source | Emulsan[a] (mg) | APO-emulsan[b] (mg) | Polysaccharide[c] (mg) |
|---|---|---|---|---|
| RAG-1 | Ethanol | 92±6 | 43±3.2 | 15±1.5 |
| | C11 | 54±3.6[d] | 8±2 | 5±1 |
| | C14 | 107±2.5 | 62±1 | 13±0.5 |
| | C16 | 83±5.2 | 38±3 | 15±1.5 |
| | C18 | 87±7.5 | 59±5.5 | 13±1.5 |
| | LB | 16±1 | 10±1 | 2±2 |
| 62C | C11 | 22±3.2* | 8±2.6 | 3±1* |
| | C14 | 11±2* | 6±3* | 5±2* |
| | C16 | 34±4.1* | 10±1* | 7±1* |
| | C18 | 44±5* | 30±4.3* | 11±3* |
| | LB | 18±1** | 5±2* | 2±0 |
| 13D | C11 | 52±3.6 | 8±3 | 5±3 |
| | C14 | 45±3.6* | 11±2.6* | 8±0.7* |
| | C16 | 45±4.3* | 5±2* | 2±1.7* |
| | C18 | 45±5* | 17±4* | 11±3.7 |
| | LB | 39±5.5** | 10±4 | 2±0.5 |
| 52D | C11 | 53±5.5 | 6±1.5 | 3±0.5* |
| | C14 | 72±4.7* | 8±2.5* | 4±2* |
| | C16 | 26±2* | 5±1.5 | 3±1.5* |
| | C18 | 44±2.6* | 6±1.6 | 2±0.5* |
| | LB | 28±3.5** | 8±1.5 | 2±2 |
| VRBS1 | C11 | 18±2.5* | 7±3 | ND[e] |
| | C14 | 27±1* | 4±1.5* | 3±1* |
| | C16 | 32±4.5* | 10±1.5* | 1±0.5* |
| | C18 | 48±8* | 13±3.5* | 5±2* |
| | LB | 28±0.5** | 8±0.5* | 2±0.5 |

[a] Emulsan at 6 days of culture after ether extraction to remove unbound FAs
[b] Apo-emulsan after phenol extraction to remove noncovalently bound proteins
[c] Polysaccharide backbone after alkaline hydrolysis to remove covalently bound FAs
[d] Standard deviation of the mean from three replicates, $P < 0.05$ compared to *A. calcoaceticus* RAG-1
[e] Not detected
* Significant decrease compared to *A. calcoaceticus* RAG-1, $P < 0.05$
** Significant increase compared to *A. calcoaceticus* RAG-1, $P < 0.05$

fed FA source. In the case of 62C, a high content of C16 (65, 89, and 42 mol%) was found when grown on LB+C11, LB+C14, and LB+C16, respectively (Table 3). Mutant VRBS1 generated emulsan with a high content of C19: 12 nmol (43 mol%) when grown on LB+C14, implying direct incorporation (data not shown). In the case of the parent strain and transposon mutants, low FA contents were observed when grown on LB only (Fig. 1), consistent with the low yields of polymer in Table 2.

## Molecular weight

Molecular weight of the polysaccharide backbone ranged from $1.38\times10^5$ to $6.97\times10^5$ Da for all of the emulsans generated from the mutants, compared with $1.90\times10^5$ to $7.63\times10^5$ Da for the parent strain (data not shown). Polydispersity ranged between 1.29 and 1.61 for the parent strain and 1.01 and 1.65 for the mutants (data not shown). *A. calcoaceticus* RAG-1 grown on ethanol generated molecular weights of $1.78\times10^5$, compared to $6.97\times10^5$ Da in the case of mutant 62C grown on C14. A low molecular weight was also observed in the case of the parent strain grown on LB.

## Emulsification behavior

The emulsification assay provided a quantitative estimate of solution behavior. The parent strain, *A. calcoaceticus* RAG-1, exhibited the highest emulsification activity of the emulsans, with the sample generated by growth on LB+C18 producing 330 Klett units (assayed on *n*-dodecane) as compared to 71, 20, 75 and 28 observed for 52D, 62C, 13D and VRBS1, respectively (Table 4). For mutant 13D, the emulsification properties of the emulsans generated by growth on LB+C16 exhibited the highest emulsification capacity of any sample in the study: 338 Klett units on *n*-tetradecane, compared to 176, 13, 28 and 0 for *A. calcoaceticus* RAG-1, 52D, 62C and VRBS1, respectively (Table 4). The emulsan from 52D exhibited reduced emulsification activity as the carbon chain length of the oil substrate to be emulsified decreased, a similar feature observed with the emulsan from the parent strain except when it was grown on LB+C18.

The mutants 13D and 52D (when grown on LB plus C16 and LB plus C14, respectively) showed 338 (assayed on *n*-tetradecane) and 306 (assayed on *n*-hexadecane) Klett units, respectively, while the parent strain had only 176 and 24 Klett units, respectively. A large mole percentage of C16 (48 mol%) and C18 (42 mol%) was found in 13D and 52D, respectively, as compared to the parent strain where only 27 mol% of C16 and no C18 was observed. In addition, even 52D when grown on LB+C14, with no incorporation of 2- or 3-OH, had 306 Klett units of activity.

**Table 3** FA content for emulsans produced by *A. calcoaceticus* RAG-1 (column 1) and the transposon mutants 62C (column 2), 13D (column 3), 52D (column 4) and VRBS1 (column 5)[a]

| Emulsan FA | FA composition (nmol/mg of emulsan) Carbon source | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LB+C11 | | | | | LB+C14 | | | | | LB+C16 | | | | | LB+C18 | | | | |
| substituents | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 |
| C12 | 24±3.5 | 1.5*±0.5 | 3*±2.6 | –[b] | – | 105±4 | – | 12*±1 | – | – | 288 ±10 | 4* ±2.3 | 4* ±3.7 | 2*±0.5 | – | 41±6.8 | – | – | 131**±6.4 | – |
| C 12:O,2OH | 33±5.8 | – | 1.5*±1.5 | – | – | 109 ±9.5 | – | 3.5* ±1.8 | – | – | – | 19** ±1.7 | 14**±1.5 | 48** ±5.5 | – | 13 ±0.5 | – | 2* | 63**±6 | – |
| C14 | 4±1.7 | 15±1.5 | 33**±2.6 | 0.5*– | – | 315±17 | – | – | 3*– | – | – | 22**±4.5 | 27**±5.2 | 3**– | – | 34 ±9.5 | – | – | 21*±1 | – |
| C16 | 6±2 | 156**±4.5 | 4±2.6 | 0.5*±0.5 | – | 72±7 | 16*±2.6 | 76±9.6 | 1.5*±1.3 | 6*±2.3 | 184±9 | 65* ±7 | 75*±4.5 | – | – | 7– | – | 9±0.5 | 24**– | – |
| C17 | 37±7 | 2*±0.7 | 4*± | – | – | 3– | – | – | – | – | – | – | 29**±5.5 | – | – | – | 45**±0.5 | – | – | – |
| C18 | 88±7.6 | – | 20*±2.5 | – | – | – | – | 18**±5.5 | 5**±1.5 | 11**±1.5 | 5±1.7 | – | – | 4±2 | – | 3– | – | – | 13**±5 | – |
| C19 | 69±8 | 48*±5 | 24*±5.5 | – | – | – | – | – | – | 12**±1.6 | – | – | – | – | – | 26±1.5 | 32±3.7 | – | 12*±1.6 | – |

[a] Peaks observed for typical fragmentation patterns of FAs based on GC-MS analysis. Some additional peaks were not identifiable based on comparisons to mass spectra of standard FA methyl esters, spectra in the instrument data bank or mass spectra libraries available on the Internet

[b] Not detected

* Significantly decreased as compared to *A. calcoaceticus* RAG-1, $P < 0.05$

** Significantly increased as compared to *A. calcoaceticus* RAG-1, $P < 0.05$

**Table 4** Emulsification behavior of emulsans from the parent strain and transposon mutants when assayed on three different alkanes (hexadecane, tetradecane and dodecane)[a]

| Strain | Carbon source | Activity (Klett Units) | | |
|---|---|---|---|---|
| | | *n*-Hexadecane | *n*-Tetradecane | *n*-Dodecane |
| RAG-1 | Ethanol | 121±25 | 65±5 | 50±13 |
| | LB+C11 | N.D.[b] | N.D. | N.D. |
| | LB+C14 | 24±0.5 | N.D. | 11±3 |
| | LB+C16 | 260±10 | 176±3 | 88±3 |
| | LB+C18 | 75±15 | 136±15 | 330±26 |
| 62 C | LB+C11 | 10±1** | 5±0.5** | 8±3** |
| | LB+C14 | 17±2.5* | 13±3** | 5±0* |
| | LB+C16 | 10±5* | 28±7* | 40±13* |
| | LB+C18 | 20±9* | 11±3* | 20±5* |
| 13 D | LB+C11 | 51±8* | 13±3** | 31±10** |
| | LB+C14 | N.D.* | N.D. | N.D.* |
| | LB+C16 | 196±15* | 338±25** | 251±28** |
| | LB+C18 | 105±18** | 101±23* | 75±13* |
| 52 D | LB+C11 | 16±17** | 16±3** | 5±0** |
| | LB+C14 | 306±20** | 250±30** | 151±33** |
| | LB+C16 | 70±13* | 13±3* | 5±0* |
| | LB+C18 | 216±25** | 206±25** | 71±10* |
| VRBS-1 | LB+C11 | N.D. | N.D. | N.D. |
| | LB+C14 | 18±10 | 31±15** | 30±22 |
| | LB+C16 | N.D.* | N.D.* | N.D.* |
| | LB+C18 | 71±25 | 35±15* | 28±7* |

[a] Average and standard deviation for three replicates

[b] Not detected

* Significant decrease when compared to *A. calcoaceticus* RAG-1, $P < 0.05$

** Significant increase when compared to *A. calcoaceticus* RAG-1, $P < 0.05$

## Discussion

Mutants were screened for direct incorporation of exogenous FAs into emulsan in order to attain more control over the structure of the bioemulsifier. Theoretically, modifying the structure of emulsan would influence how the polymer performs as a surfactant/emulsifier due to alterations in amphiphilic behavior. With the ability to tailor the structure, our goal is to develop a correlation between bioemulsifier structure (types and extent of FA substituents) and function (emulsifying activity).

The transposon mutants, unlike the parent strain, *A. calcoaceticus* RAG-1, were unable to grow on defined media supplemented with ethanol, and only VRBS1 was capable of growth on minimal medium with FAs as a sole carbon source. The inability of the transposon mutants to grow on minimal medium made comparisons to prior studies with *A. calcoaceticus* RAG-1 (Gorvenko et al. 1997; 1999; Zhang et al. 1997) difficult, necessitating the use of LB in this study.

A number of differences were observed when the yield of emulsan from mutants was compared with that of the parent strain, e.g., mutant 52D grown on LB+C14 produced the maximum amount of emulsan (obtained after ether extraction) compared to the other mutants grown on different carbon sources but the amount was

still less as compared to *A. calcoaceticus* RAG-1. Of the mutants, 62C grown on LB+C18 generated a maximum yield of the emulsan but the amount remained less than that produced by *A. calcoaceticus* RAG-1. Yields of emulsan from the parent strain and the mutants grown on LB alone were very low, although yield depended on the type of FA used to supplement the LB. From these data, it is possible to influence emulsan production from the transposon mutants through the use of different carbon sources.

The reasons for the variation in FA profiles of emulsans from the transposon mutants are not immediately apparent. Since the complex LB medium supplies the metabolites required for growth, metabolites the mutants are unable to produce themselves, the subcellular concentrations of these metabolites may play an indirect role in the availability of FAs for incorporation into emulsan. One of the more significant determinants of FA chain lengths in *E. coli* appears to be the availability of malonyl-CoA (Magnusen et al. 1993) and competition for its use in either FA initiation or terminal elongation. Since both metabolites affected by mutants VRBS1 and 52D could potentially affect malonyl-CoA synthesis (biotin in its conversion from acetyl-CoA; cysteine as required for the biosynthesis of coenzyme A from pantothenate), the general response is a smaller pool of FAs available to modify the emulsan polysaccharide main chain. An examination of total cellular FAs and comparison with those from emulsan in *A. calcoaceticus* RAG-1 and mutants may reveal the effects of these metabolic changes on FA profiles, both endogenous and exogenous.

No mutants were identified with direct disruption of β-oxidation pathways, a feature that may provide further control of FA substitution patterns. The generally lower level of FA activation leading to acylation, reflected in the lower degree of substitution (nmol FA/mg emulsan) when compared with the prior studies on minimal media suggests a general inhibition in metabolic activity, likely due to the lower charging of pathways involved in FA metabolism. The higher percentage of C14, C16, and C18 appended onto the polysaccharide backbone in some cases by the transposon mutants may reflect substrate preference for this chain length of FAs by the acyl transferase involved. It is interesting to note that incorporation of C22 was observed only when *A. calcoaceticus* RAG-1 was grown on LB plus C11 (data not shown). However, incorporation of C22 was not observed in any of the mutants.

In previous physiology experiments with the parent strain using *n*-alkanoic acid carbon sources of varying chain length, the emulsification specificity of the resulting structurally different emulsans was altered (Gorvenko et al. 1997). Specifically, by incorporating relatively longer side chain substituents such as C17:0 and C17:1 into emulsans, the polymer demonstrated increased specificity for dispersing oil phases which consisted of longer chain length *n*-alkanes. The inability of the emulsan derived from *A. calcoaceticus* RAG-1 and 13D to emulsify C11 and C14, respectively, suggests that there is a lower threshold of substitution by FAs required to maintain emulsification properties, since these polymers contained only 186 and 129 nmol FA/mg emulsan, respectively. Presumably the required balance of hydrophobic/hydrophilic components on the polymer to maintain oil-in-water emulsions cannot be met once the degree of FA substitution falls below these thresholds.

It was reported that emulsan with C12 (2-and 3-OH) substituents played an important role in the emulsification behavior (Belsky et al. 1979). However, in another study (Zhang et al. 1997), emulsan analogs containing high proportions of C12, 2-OH, 3-OH did not show enhanced emulsifying activity. In this work, we also investigated whether any particular type of FA (hydroxylated, saturated or unsaturated and long chain) had an effect on emulsification activity. From the results, the correlation between FA composition and emulsification activity is complex. Higher emulsification activities were observed in mutants 13D and 52D though there was no, or at most a lower mole percentage, of 2- or 3-OH present. In the case of mutants 13D and 52D grown on C16 and C14, respectively, enhanced emulsification activity (assayed on *n*-tetradecane and *n*-hexadecane, respectively) was observed as compared to the parent strain grown on the same carbon source. Long chain FA composition and a large mole percentage of C16 (48 mol%) and C18 (42 mol%) play a role in the emulsification behavior in the mutants. No discernable effect on surface tension and interfacial tension was noted for the emulsan variants (data not shown).

Although emulsan does not reduce interfacial tension as effectively as small molecule surfactants, it is believed that emulsans bind tightly to the surface of oil droplets and thereby prevent coalescence (Gutnick and Shabtai 1987). This property has been attributed to its amphiphilic structure, which provides multiple anchoring sites (the hydrophobic side chains) to the dispersed phase. In this study, a mutagenesis approach was employed successfully to generate a new family of emulsan structural analogs. These polymers retained emulsification behavior while also containing significantly reduced levels of FA substitution. The results provide important insight into the bioengineering of bioemulsifier-producing microorganisms and provide a path toward highly tailored structures potentially useful in environmental, biomedical, and personal care applications.

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