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D. Örnek · A. Jayaraman · B.C. Syrett · C.-H. Hsu
F.B. Mansfeld · T.K. Wood

Pitting corrosion inhibition of aluminum 2024 by *Bacillus* biofilms secreting polyaspartate or γ-polyglutamate

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Abstract Pitting corrosion of aluminum 2024 in Luria Bertani medium was reduced by the secretion of anionic peptides by engineered and natural *Bacillus* biofilms and was studied in continuous reactors using electrochemical impedance spectroscopy. Compared to sterile controls, pitting was reduced dramatically by the presence of the biofilms. The secretion of a 20 amino acid polyaspartate peptide by an engineered *Bacillus subtilis* WB600/pBE92-Asp biofilm slightly reduced the corrosion rate of the passive aluminum alloy at pH 6.5; however, the secretion of γ-polyglutamate by a *Bacillus licheniformis* biofilm reduced the corrosion rate by 90% (compared to the *B. subtilis* WB600/pBE92 biofilm which did not secrete polyaspartate or γ-polyglutamate). The corrosion potential (E_{corr}) of aluminum 2024 was increased by about 0.15–0.44 V due to the formation of *B. subtilis* and *B. licheniformis* biofilms as compared to sterile controls. The increase of E_{corr} and the observed prevention of pitting indicate that the pitting potential (E_{pit}) had increased. This result and the further decrease of corrosion rates for the passive aluminum alloy suggest that the rate of the anodic metal dissolution reaction was reduced by an inhibitor produced by the biofilms. Purified γ-polyglutamate also decreased the corrosion rates of aluminum 2024.

D. Örnek · A. Jayaraman · T.K. Wood (✉)
Departments of Chemical Engineering & Molecular and Cellular Biology, University of Connecticut, Storrs, CT 06269–3222, USA
e-mail: twood@engr.uconn.edu
Tel.: +1-860-486-2483, Fax: +1-860-486-2959

B.C. Syrett
Electric Power Research Institute, Palo Alto, CA 94304–1395, USA

C.-H. Hsu · F.B. Mansfeld
Materials Science & Engineering, University of Southern California, Los Angeles, CA 90089, USA

Present address:
A. Jayaraman, Center for Engineering in Medicine, Mass. General Hospital and Harvard Medical School, Boston, MA 02114, USA

Nomenclature A: area of the metal exposed to the medium in the reactor, cm^2 · A_{pit}: pitted area, cm^2 · E: corrosion inhibition efficiency, 100 $(C_o–C_i)/C_o$ · E_{corr}: corrosion potential, mV · E_{pit}: pitting corrosion potential, mV · E_{rel}: relative inhibition efficiency, $100(1\text{-}R^o_{p,\,st}/R^o_{p,\,bio})$ · i_{corr}: corrosion current density, B/R_p, B is a constant · R_p: polarization resistance · $1/R_p$: approximate corrosion rate · C_i: corrosion rate with inhibitor, Ω^{-1} · C_o: corrosion rate without inhibitor, Ω^{-1} · R^o_p: R_p.A, $\Omega.cm^2$ · $R^o_{p,bio}$: R^o_p value determined in media containing bacteria · $R^o_{p,\,st}$: R^o_p value determined in sterile media · R_{pit}: polarization resistance of growing pit, Ω · R^o_{pit} · : $R_{pit}.A_{pit}$, $\Omega.cm^2$

Introduction

Electrochemical impedance spectroscopy (EIS) has been used to show that protective biofilms on metal surfaces decrease the corrosion rate of SAE 1018 steel by 98% (Jayaraman et al. 1997), aluminum 2024 by 86% (Jayaraman et al. 1999b), and copper by 95% (Jayaraman et al. 1999b) compared to sterile controls in continuous reactors. It was suggested that the bacteria provided the observed corrosion protection by consuming dissolved oxygen before it can reach the metal surface and take part in the corrosion reaction. It has also been demonstrated that the corrosive attack on stainless steel by sulfate reducing bacteria (SRB) can be reduced if protective biofilms are engineered to secrete antimicrobial peptides such as indolicidin and bactenecin, which kill corrosion-causing SRB (Jayaraman et al. 1999a).

Purified anionic organic compounds have been shown to reduce the corrosion rates of steel, copper, and aluminum. Sekine et al. (1992) showed that the corrosion inhibition efficiency E [$100(C_o–C_i)/C_o$, where C_i and C_o are, respectively, the corrosion rates with and without the inhibitor present] of anionic polymers for mild steel depends on the molecular weight and number of carboxylic acid groups in a solution containing Ca^{2+} or $Cl^–$. Hefter

et al. (1997) reported that straight chain aliphatic monocarboxylates (n=6–10 where n is the number of carbon atoms) and dicarboxylates (n=3–14) in a mildly saline solution at pH 8.4 decreased the corrosion rate of mild steel by as much as 99.9% and of copper by 99% with the corrosion inhibition efficiency (E) depending on the chain length. These carboxylates also increased the pitting corrosion potential E_{pit} of aluminum which is the potential above which pits can initiate and grow (Hefter et al. 1997). Mueller et al. (1993) reported that the addition of a 20 amino acid peptide of purified polyaspartate at 10 µg/ml slightly reduced the corrosion rate of mild steel 1018 in fresh or brackish waters at pH 8.2, while polyaspartate at 100 µg/ml slightly reduced the corrosion rate of mild steel in seawater.

Aluminum and its alloys are used in the process industry where better corrosion resistance is required (Fontana 1986). The most common form of attack of aluminum and its alloys in aerated, near-neutral solutions containing chloride is pitting corrosion (Hefter et al. 1997), which is a form of localized corrosion on the metal surface that can cause equipment to fail with only a small percentage weight loss of the entire structure (Fontana 1986). Although the addition of some inhibitors, such as hydroxide, chromate or silicate salt, is sometimes helpful, complete protection against pitting corrosion in these aluminum alloys is difficult (Fontana 1986).

Bacillus licheniformis ATCC 9945A produces large amounts of γ-polyglutamate that is part of its capsule and is freely secreted into the growth medium (Ko and Gross 1998). γ-Polyglutamate is a homopolymer of glutamic acid that has amide linkages between the glutamate γ-carboxyl and α-amino groups (Ko and Gross 1998). Depending on nutrients, pH, and aeration rate, the yield of γ-polyglutamate is between 5 and 23 g/l, and its molecular weight ranges from 1 million to 5 million g/mol (Cromwick et al. 1996; Ko and Gross 1998). Mclean et al. (1990) investigated the metal-binding affinity of γ-polyglutamate produced by *B. licheniformis* and found that Cu^{2+}, Al^{3+}, Cr^{3+}, and Fe^{3+} have great affinity for the *B. licheniformis* capsule.

The goal of this study was to evaluate whether genetically engineered bacteria that secrete biological molecules can prevent pitting corrosion of aluminum 2024 in Luria Bertani (LB) medium. In addition, the impact of γ-polyglutamate on decreasing pitting corrosion as well as uniform corrosion of passive aluminum 2024 was investigated. This is the first report of using an engineered biofilm to secrete a general corrosion inhibitor.

Materials and methods

Bacterial strains, plasmids, and growth media

Escherichia coli XL1-Blue was purchased from Stratagene (La Jolla, Calif.). The γ-polyglutamate-producing strain *B. licheniformis* was obtained from ATCC (Manassas, Va., strain 9945A) and formed highly mucoid colonies. *Bacillus subtilis* WB600 (Wu et al. 1991) was obtained from Dr. Sui-Lam Wong of the University of Calgary and is a protease-deficient strain. It was transformed with pBE92, a derivative of pBE60 (Vasantha et al. 1992), to become resistant to 50–100 µg/ml kanamycin and was used as a biofilm-forming negative control that does not secrete a corrosion inhibitor. To preserve the bacteria indefinitely at –80°C (Maniatis et al. 1982), 0.15 ml of sterile glycerol was added to 0.85 ml of overnight bacterial culture grown in LB medium (Maniatis et al. 1982).

Plasmid pBE92 contains the constitutive alkaline protease (APR) promoter, APR signal sequence, and the alkaline phosphatase reporter gene; it was obtained from du Pont de Nemours (Wilmington, Del.). Artificial seawater was Väätänen nine salts solution (VNSS, pH 7.5) (Hernandez et al. 1994). One percent NaCl with 100 µg/ml kanamycin was used to test the effect of purified γ-polyglutamate on the corrosion reduction of aluminum in a batch reactor. Polyaspartate was synthesized as a ten amino acid polymer by Genosys Biotechnologies (Woodlands, Tex.) at 76% purity.

Plasmid construction and transformation of *E. coli* and *B. subtilis*

B. subtilis WB600 was engineered to constitutively secrete either polyaspartate or polyglutamate as 20 amino acid polymers by using recombinant DNA methods as described by Maniatis et al. (1982) and Rodriguez and Tait (1983). Plasmid DNA was isolated from *Bacillus* strains according to the procedure of Bramucci and Nagarajan (1996). Plasmids pBE92-Asp and pBE92-Glu were designed for efficient secretion of the inhibitors polyaspartate and polyglutamate, respectively, under control of the constitutive APR promoter (Vasantha et al. 1984) as 20 amino acid peptides fused to the 30 amino acid *B. amyloliquefaciens* APR signal sequence (Chen and Nagarajan 1993). The APR signal sequence of *B. amyloliquefaciens* was slightly altered in pBE92 as glutamine was replaced with serine at the –2 position of the cleavage site (correct signal processing still occurs; Chen and Nagarajan 1994), and methionine, the initiation codon, was replaced with valine at the –30 position (an alternative start codon; Snyder and Champness 1997). An *Eco*RI site was also engineered downstream of the stop codon for each construct to introduce a unique site into pBE92-Asp and pBE92-Glu. Figure 1 gives the two sets of synthetic DNA oligos for the polyaspartate gene and for the polyglutamate gene which were synthesized by Gibco-BRL Life Technologies (Long Island, N.Y.) with flanking *Nhe*I and *Hin*dIII restriction enzyme sites and with an additional six bases at either end for efficient restriction. For each gene, the two fully complementary oligos of each construct were annealed, and the complementary regions completed using *Taq* polymerase (one cycle, 30 s at 94°C, followed by 30 s at 55°C, and 2 h at 72°C) with a Perkin-Elmer thermal cycler N801–0150 (Perkin Elmer, Norwalk, Conn.). Each of the full-length genes was then digested with *Nhe*I and *Hin*dIII and ligated into plasmid vector pBE92 isolated from *E. coli* XL1-Blue and digested with *Nhe*I, *Hin*dIII, and *Sal*I simultaneously (a *Sal*I site is present in the removed alkaline phosphatase gene).

E. coli XL1-Blue transformants containing the correct insert (pBE92-Asp or pBE92-Glu) were screened as white colonies on LB agar plates containing 100 µg/ml ampicillin and 40 µg/ml 5-bromo-4-chloro-3-indolyl phosphate; transformants with the correct insert produced white colonies while the reclosed vector resulted in blue colonies. *E. coli* XL1-Blue cells were made electrocompetent according to the method of Smith and Iglewski (1989) and electroporated using a gene pulser/pulse controller (Bio-Rad, Hercules, Calif.). *B. subtilis* WB600 strains were transformed with the two plasmids according to the two-step method of Cutting and Vander Horn (1990) and plated on LB agar plates containing 50 µg/ml kanamycin. pBE92-Asp and pBE92-Glu plasmids were isolated from *E. coli* XL1-Blue and *B. subtilis* WB600, digested with *Nhe*I, *Hin*dIII, and *Eco*R1 restriction enzymes, and verified using horizontal gel electrophoresis.

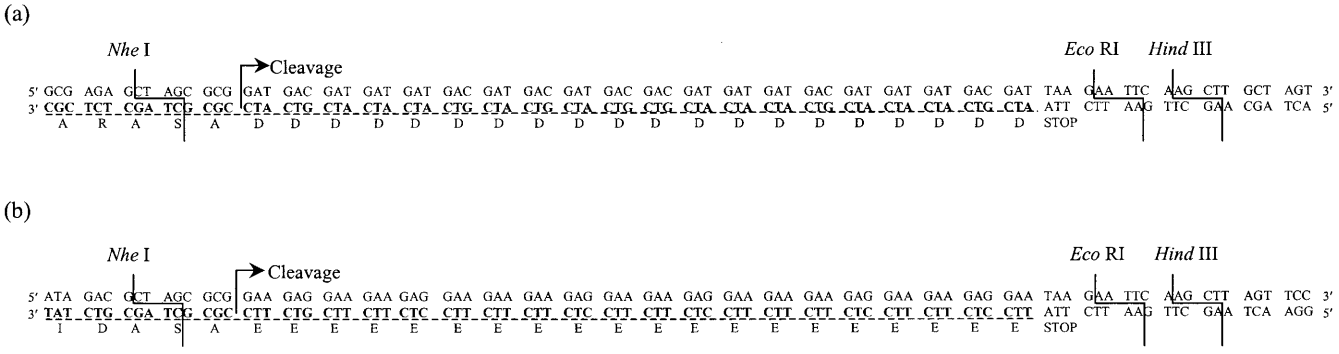

Fig. 1 Oligonucleotides used for cloning **a** polyaspartate and **b** polyglutamate as fusions to the alkaline protease (APR) leader sequence. The *bold* nucleotides with a *dashed line* indicate bases filled by *Taq* polymerase. *S* Serine, *A* alanine, *D* aspartate, *E* glutamate, *I* isoleucine. Only the relevant restriction sites are shown. *ASA* indicates the last three amino acids of the APR leader

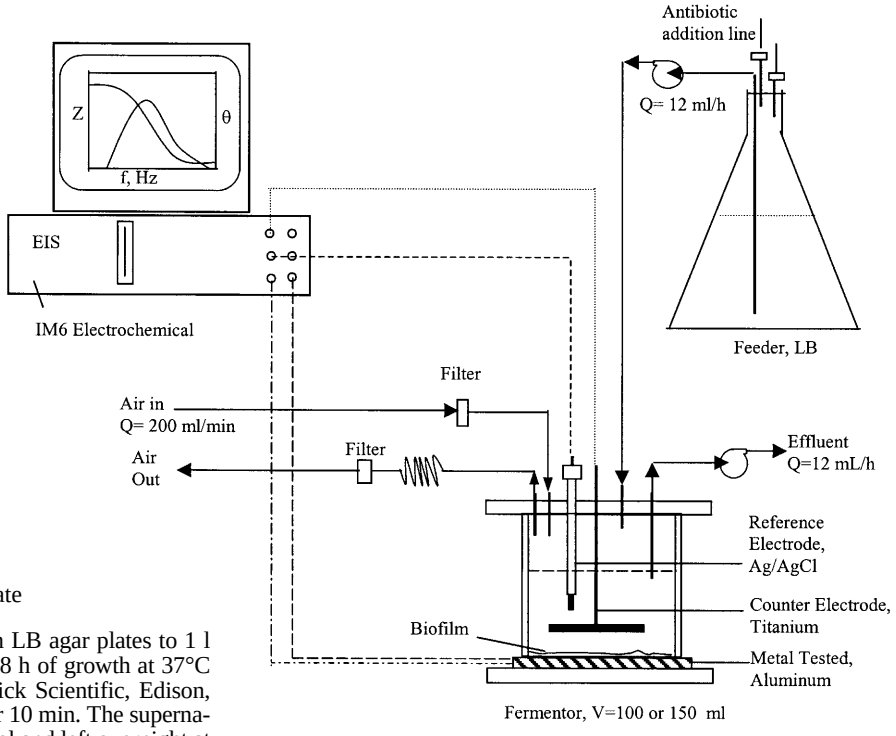

Fig. 2 Continuous fermentor interfaced to the electrochemical impedance spectrometer

Preparation and purification of γ-polyglutamate

Highly mucoid colonies were transferred from LB agar plates to 1 l flasks containing 0.5 l of LB medium. After 48 h of growth at 37°C and 250 rpm (series 25 shaker, New Brunswick Scientific, Edison, N.J.), the cells were centrifuged at 15,000 *g* for 10 min. The supernatant was combined with four volumes of ethanol and left overnight at –20°C. The precipitated capsule was concentrated by centrifugation at 15,000 *g* for 20 min at –20°C and resuspended in a minimal volume of water. Proteins were removed by two successive 1-h incubations of the capsule with pronase (5 µg/ml) at 37°C. The capsule was combined with four volumes of ethanol and left overnight at –20°C. The capsule was precipitated at 15,000 *g* for 10 min at 4°C and dissolved in 10 ml water. The suspended capsule was frozen quickly in dry ice, and then desiccated thoroughly while frozen in a freeze dry system (Labconco, Kansas City, Mo.). The concentration of purified exopolymer solution was measured by weighing the freeze-dried γ-polyglutamate before it was dissolved in water.

TLC of the hydrolysis product

Purified γ-polyglutamate was hydrolyzed with 6 N HCl for 1 or 3 days at 100°C to produce glutamate (Hara et al. 1982; Braithwaite and Smith 1985). Thin-layer chromatography (TLC) of the hydrolysis products was carried out by the ascending technique on a 200 µm silica gel plate (Selecto Scientific, Ga.) at room temperature for 2.5 h (Hara et al. 1982; Braithwaite and Smith 1985). The solvent system used was 4:1:1 *n*-butanol:acetic acid:water. After development and drying, the spots were detected by spraying with methanol containing 0.4% ninhydrin.

Metal coupon preparation for testing and post-test examination

Squares (10 cm) of mild steel 1010 (UNS G10100) and aluminum alloy 2024 (UNS A92024) (Yarde Metals, Bristol, Conn.) were cut from sheet stock (1.2 mm thickness) and polished with 240 grit polishing paper (3M, St. Paul, Minn.). At the end of the experiment, the metal surface was cleaned under tap water with vigorous scrubbing using a rubber stopper. An FD Mavica digital still camera (Sony) was used to take a digitalized photograph of the sample surface. It was converted to a black and white image where pitted areas were black and the rest white. Photoshop software was used to calculate the proportion of black and white pixels.

Continuous culture corrosion experiments

Autoclavable continuous reactors (Fig. 2) consisting of a 5.5 or 6.0 cm diameter glass cylinder on top of the metal sample were

used as described previously (Jayaraman et al. 1997). The working volume of the reactor was 100 ml or 150 ml with an airflow rate of 200 ml/min (monitored with a FM1050 series flowmeter, Matheson Gas Company, Cucamonga, Calif.). The growth temperature was maintained at 30°C by heating tape wrapped around the reactor. Sterile medium used in each experiment was fed at a nutrient flowrate of 12 ml/h using a Masterflex precision standard drive with a 10-turn potentiometer (Cole-Parmer, Niles, Ill.); hence, the dilution rate was 0.12/h for the 100 ml reactor and 0.08/h for the 150 ml reactor. The reactors were inoculated with overnight cultures of *B. subtilis* WB600 strains grown from –80°C glycerol stocks in 250 ml shake flasks with 25 ml LB medium supplemented with 50 µg/ml kanamycin at 37°C and with shaking at 250 rpm (series 25 shaker, New Brunswick Scientific, Edison, N.J.). *B. licheniformis* was grown without antibiotics. Sterile control experiments were conducted with 100 µg/ml kanamycin to ensure sterility. Biofilms were allowed to develop for 15–18 h for *B. subtilis* and 3 days for *B. licheniformis* in batch mode then nutrients were added continuously (100 µg/ml kanamycin was added continuously for plasmid retention for the *B. subtilis* cultures). All continuous reactor experiments were conducted at least in duplicate.

Electrochemical impedance spectroscopy

Biofilm development and corrosion behavior were monitored during the continuous reactor experiments using EIS as described previously (Jayaraman et al. 1997; Mansfeld et al. 2000; Örnek et al. 2001). EIS is a non-destructive technique that can be used to monitor the corrosion behavior as a function of exposure time without disturbing the properties of the metal surface under investigation or the biofilm. The EIS data were collected at the corrosion potential (E_{corr}) using an IM6 impedance spectrometer (Bioanalytical Systems-Zahner, West Lafayette, Ind.) interfaced to a Gateway Pentium GP6 300 MHz computer (North Sioux, S.D.). Impedance spectra were obtained approximately every 24 h. An autoclavable Ag/AgCl electrode was used as the reference electrode. All potential values reported here refer to the Ag/AgCl electrode (+0.209 V versus the standard hydrogen electrode).

The experimental impedance spectra were analyzed using equivalent circuits (EC) which have been proposed by Mansfeld and co-workers (1992b) to describe the impedance behavior of aluminum and mild steel. For cases of uniform corrosion, the polarization resistance R_p is obtained from this analysis from which the corrosion current density, i_{corr}, can be estimated using the Stern-Geary equation $i_{corr} = B/R_p$, where B is a constant. The corrosion current density can be converted into a corrosion rate using Faraday's law (Mansfeld 1976). In the present screening tests, the values of $1/R^o_p$, which is the reciprocal of the product of R_p and the area A of the metal exposed to medium in the reactor, were used as an estimate of relative corrosion rates. By considering the changes of R^o_p and E_{corr}, it is possible to suggest mechanisms by which bacteria change the corrosion behavior of different materials in different media. In cases where pitting of aluminum is observed, the pitting model proposed by Mansfeld et al. (Mansfeld and Shih 1988; Mansfeld et al. 1989, 1992a; Shih and Mansfeld 1989) can be used to determine R_p for the passive surface and the polarization resistance R_{pit} of growing pits. R_{pit} can be normalized by the time dependent value of the pitted area A_{pit} to obtain $R^o_{pit} = R_{pit} \times A_{pit}$. This procedure has recently been demonstrated by Örnek et al. (2001) for pitting of aluminum 2024 in artificial seawater and its inhibition by *Bacillus subtilis* (see also Mansfeld et al. 2000). In the present study, which evaluates corrosion inhibition of aluminum 2024 by *Bacillus* biofilms secreting polyaspartate or γ-polyglutamate, only the values of R^o_p are reported.

Results

Duplicate results obtained with continuous sterile reactors demonstrated that the addition of the ten amino acid polymer of purified polyaspartate at 20 µg/ml to artificial seawater increased R_p for mild steel from 9,600± 2,600 Ω-cm^2 to 26,000±3,000 Ω-cm^2 resulting in a relative inhibition efficiency E_{rel} = 63%. $E_{rel} = 100(1–R^o_{p,st}/R^o_{p,bio})$, where $R^o_{p,st}$ and $R^o_{p,bio}$ refer to the R^o_p values determined in sterile media and in media containing bacteria, respectively. Since polyaspartate inhibits uniform corrosion as indicated previously by Mueller et al. (1993), *B. subtilis* WB600 was engineered to secrete polyaspartate. For comparison, *B. subtilis* WB600 was also engineered to secrete polyglutamate, and *B. licheniformis* ATCC 9945 A was used to produce γ-polyglutamate, which also has a repeated carboxylic acid group. *B. subtilis* WB600/pBE92 was used as a control that was not engineered to secrete corrosion inhibitors, but produces a biofilm on metal surfaces.

Corrosion inhibition of aluminum 2024 with biofilms

Pitting was observed uniformly over the aluminum surface (200–300 pits) after exposure to sterile LB medium for only 1 day (see also Mansfeld et al. 2000; Örnek et al. 2001). Using a digital camera and image software, the fraction F of the pitted surfaces at the end of the experiment was estimated to be 1.69% for a sample with 7 day exposure and 6.83% for a sample with 10 day exposure. In the presence of all biofilms, pitting was greatly reduced, as indicated by the capacitive nature of the impedance spectra (Mansfeld et al. 2000; Örnek et al. 2001) and visual observation that showed there was almost no pitting (4–5 pits) in the presence of the *Bacillus* biofilms after 7–10 days.

Fig. 3 shows the time dependence of the relative corrosion rate, $1/(R^o_p)$, for aluminum 2024 during exposure to *Bacillus* biofilms in LB medium for 4–10 days. When the biofilm was engineered to secrete polyaspartate as a 20 amino acid polymer using *B. subtilis* WB600/pBE92-Asp, there was a 2.2-fold (E_{rel} =55%) additional benefit as compared to the *B. subtilis* WB600/pBE92 biofilm. The secretion of polyglutamate did not yield additional corrosion protection of aluminum compared to *B. subtilis* WB600/pBE92. However, the secretion of γ-polyglutamate by *B. licheniformis* provided 9–11-fold (E_{rel} =89–91%) additional protection for 7 days compared to *B. subtilis* WB600/pBE92. After day 7, the corrosion rate of aluminum rose dramatically and, after day 9, it reached the same corrosion rate of the control *B. subtilis* WB600/pBE92 culture (Fig. 3). The levels of corrosion inhibition are higher than those reported previously by Jayaraman et al. (1999b) in which *Pseudomonas fragi* K or *B. brevis* 18–3 decreased the corrosion rate of aluminum 2024 compared to sterile Baars' medium (American Type Culture Collection medium 1249).

The time allowed for *B. licheniformis* to form a biofilm in batch mode was found to be important. When *B. licheniformis* was allowed to grow for only 15–18 h in batch mode (as opposed to 72 h) before nutrients were added continuously, the secretion of γ-polyglutamate

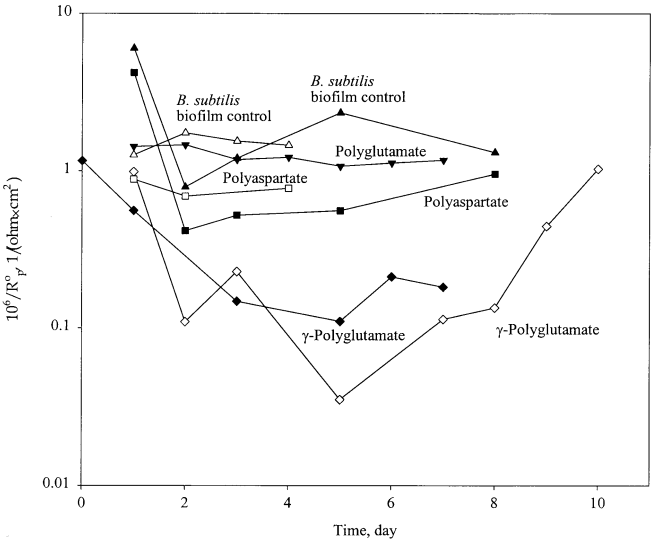

Fig. 3 Relative corrosion rates ($1/R^o_p$) of aluminum 2024 in the presence of *Bacillus subtilis* WB600 and *Bacillus licheniformis* biofilms in Luria Bertani (LB) medium at pH 6.5 in a continuous reactor. *Polyaspartate* indicates the 20 amino acid polyaspartate secreted by a *B. subtilis* WB600/pBE92-Asp biofilm, *polyglutamate* indicates the 20 amino acid polyglutamate secreted by a *B. subtilis* WB600/pBE92-Glu biofilm, and *γ-polyglutamate* indicates the anionic polymer secreted by a *B. licheniformis* biofilm. Duplicate results are shown with *open symbols*

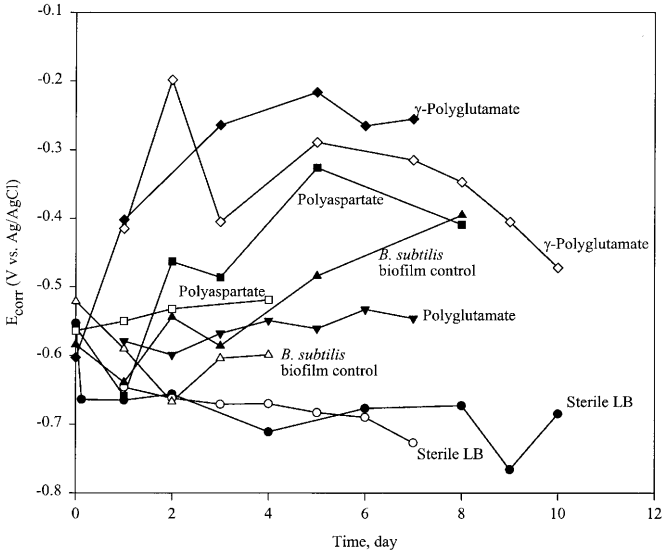

Fig. 4 Corrosion potential E_{corr} of aluminum 2024 in continuous reactors with LB medium at pH 6.5. Symbols as defined in the Fig. 2 caption

provided no more protection to aluminum 2024 than the *B. subtilis* WB600/pBE92 control.

Ennoblement of E_{corr} in the presence of biofilms

Susceptibility to pitting corrosion can be evaluated by considering the difference (ΔE) between the pitting potential (E_{pit}) and (E_{corr}) (Hefter et al. 1997). In neutral aerated media such as artificial seawater and LB medium, ΔE (E_{pit}–E_{corr}) approaches zero after a certain induction period and pitting occurs. In the presence of biofilms, E_{corr} increased to different extents during exposure to LB at pH 6.5 for 4–10 days (Fig. 4). *B. subtilis* WB600/pBE92 and *B. subtilis* WB600/pBE92-Asp biofilms increased E_{corr} by as much as 300 mV in 8 days compared to sterile controls, while *B. subtilis* WB600/pBE92-Glu increased E_{corr} by 150 mV in 7 days. *B. licheniformis* increased E_{corr} by 200–450 mV in 7–10 days. If E_{pit} would not have increased in the presence of the biofilms, these increases in E_{corr} would have caused an increase in the susceptibility to pitting. However, since the extent of pitting corrosion actually decreased significantly, E_{pit} must have increased more than E_{corr}.

The significant increase of $1/R^o_p$ after 5 days for one of the tests with biofilms producing γ-polyglutamate (Fig. 3) was accompanied by a sharp decrease of E_{corr} (Fig. 4). Further investigation showed very similar trends in a plot of log $1/R^o_p$ vs E_{corr} for the different tests with bacteria (Fig. 5). As the relative corrosion rate decreased, E_{corr} increased, suggesting that the rate

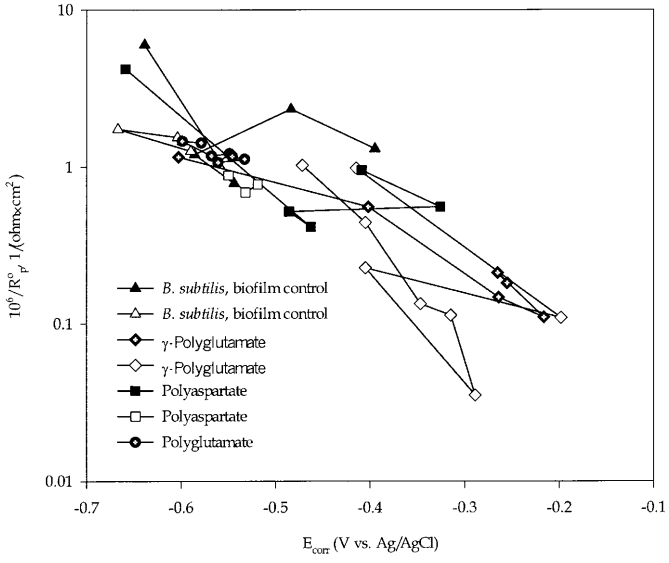

Fig. 5 Plots of log $1/R^o_p$ vs E_{corr} for the data in Figs. 2 and 3

of the anodic reaction, i.e. metal dissolution, was decreased.

γ-Polyglutamate isolation and corrosion reduction

In order to show that the extracellular product of *B. licheniformis* is primarily γ-polyglutamate and that it is this compound which is responsible for the observed 90% reduction in corrosion rates, γ-polyglutamate was purified from the supernatant of *B. licheniformis* batch cultures and then hydrolyzed with 6 N HCl so that the repeating glutamate building blocks could be detected. TLC analysis showed clearly that the hydrolysis of

γ-polyglutamic acid produced glutamic acid; note that 3-day hydrolysis gave a better spot than 24-h hydrolysis (results not shown). Thus, this experiment confirmed that the isolated polymeric compounds from the *B. licheniformis* batch reactor were made of glutamic acid, and are γ-polyglutamate. Previously, Birrer et al. (1994) showed by using nuclear magnetic resonance spectroscopy and an ultra violet (UV) and refractive index detector that *B. licheniformis* 9945A does not produce an extracellular polysaccharide product.

A spectrophotometer was also used to show the presence of γ-polyglutamate in the supernatant of a *B. licheniformis* culture. γ-Polyglutamate gave a maximum peak at UV absorption wavelength 205 nm (results not shown), as reported previously (Birrer et al. 1994).

In order to further demonstrate that γ-polyglutamate is the corrosion inhibitor responsible for the inhibition of aluminum corrosion, γ-polyglutamate was isolated and purified from *B. licheniformis* in LB medium at pH 6.5 and then tested to determine whether it inhibited aluminum corrosion. Purified γ-polyglutamate at 4.3 g/l reduced the pitting attack dramatically relative to the sterile control for which severe pitting was detected in 4–5 h. No pitting corrosion occurred in the presence of 4.3 g/l γ-polyglutamate for about 2 days and a few pits appeared on the metal surface during the 3rd day of the experiment. After 5 days, the percentage of the exposed area that was pitted (*F*) was about 2.2% in the control experiment, while in the presence of γ-polyglutamate *F* was about 1.3%. EIS analysis showed that γ-polyglutamate reduced the pitting corrosion rate of aluminum about 67% compared to the control experiment in the first 3 days of the experiment; however, it did not provide any additional protection compared to the sterile control after the 3rd day. This experiment showed that a high concentration of γ-polyglutamate successfully inhibited pit formation on the aluminum surface for only 2–3 days. γ-Polyglutamate at a lower concentration (0.1, 0.2, or 1.6 g/l) was not effective at reducing corrosion.

Discussion

The results presented here demonstrate that pitting attack of aluminum 2024 was greatly reduced in the presence of the protective biofilms. The production of polyaspartate and γ-polyglutamate by *B. subtilis* WB600 and *B. licheniformis*, respectively, resulted in additional reduction of the corrosion rate due to their carboxylic acid group. This functional group is capable of chelating or coupling aluminum ions or aluminum oxides that are present at the metal-solution interface via hydrogen bonds, dipole-dipole and Coulombic interaction (Hefter et al. 1997). Thus, the formation of an aluminum/polyaspartate or γ-polyglutamate complex may have reduced the uniform corrosion rate of aluminum 2024 compared to the rate observed in the presence of *B. subtilis* WB600/pBE92.

After 6–7 days of continuous exposure, the corrosion rate of aluminum increased steadily and there was less protection afforded by the biofilm that secreted γ-polyglutamate (Fig. 3). This reduction of corrosion protection may be due to the degradation of γ-polyglutamate by γ-polyglutamate depolymerase produced by *B. licheniformis* (He et al. 2000). Birrer et al. (1994) showed that γ-polyglutamate depolymerase is intracellularly located or cell-bound. Tran et al. (2000) pointed out that a *comQXPA* quorum-sensing system in *B. subtilis* and *B. licheniformis* plays a key role in the regulation of γ-polyglutamate depolymerase; hence, when the cell concentration in the biofilm reached a specific density, the protective γ-polyglutamate may have been degraded.

The drastic reduction in pitting attack of the aluminum coupons in the presence of the protective biofilm suggests that the biofilm, which is composed of exopolysaccharide and protein (Sutherland 1982; Neidhardt et al. 1990), might have acted to buffer the pH near pit initiation sites. DeBerry and Viehbeck (1988) reported that if the pH is low enough in a given region, biofilm anions can absorb this localized acidity. This surface pH buffering effect would thus increase E_{pit}. Since E_{pit} increases with a decrease of the activity a_{Cl^-} of chloride ions, it is also possible that the biofilms have a negative charge that would repel chloride ions and reduce a_{Cl^-} at the metal surface. The inhibition of pitting in the presence of bacteria could also be due to exclusion of oxygen from the metal surface that would reduce the rate of the cathodic reduction resulting in a decrease of E_{corr} below E_{pit} (Örnek et al. 2001). However, the results in Fig. 4 show a significant increase of E_{corr}. Since E_{corr} cannot exceed E_{pit}, the changes of E_{corr} in the presence of the bacteria demonstrate that biofilm formation has greatly increased E_{pit}. This observed increase of E_{pit} is most likely due to the formation of a very effective inhibitor by the *B. subtilis* biofilm and is enhanced by the production of additional inhibitors by the engineered biofilms. The fact that all data points in the plot of log $1/R^o_p$ vs E_{corr} fall within a small band (Fig. 5) suggests that the mechanism of corrosion inhibition is the same in all cases although the extent of this effect depends on the nature of the biofilms and the exposure time. The decrease of the uniform corrosion rates is caused by a decrease of the anodic dissolution rate of the passive aluminum alloy due to biofilm formation.

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References

Birrer GA, Cromwick A, Gross RA (1994) γ-Poly(glutamic acid) formation by *Bacillus licheniformis* 9945a: physiological and biochemical studies. Int J Biol Macromol 16:265–275

Braithwaite A, Smith FJ (1985) Chromatographic methods, 4th edn. Chapman and Hall, New York

Bramucci MG, Nagarajan V (1996) Direct selection of cloned DNA in *Bacillus subtilis* based on sucrose-induced lethality. Appl Environ Microbiol 62:3948–3953

Chen M, Nagarajan V (1993) The role of signal peptide and mature protein in RNase (barnase) export from *Bacillus subtilis*. Mol Gen Genet 239:409–415

Chen M, Nagarajan V (1994) Effect of alteration of charged residues at the N-termini of signal peptides on protein export in *Bacillus subtilis*. J Bacteriol 176:5796–5801

Cromwick AM, Birrer GA, Gross RA (1996) Effects of pH and aeration on γ-poly(glutamic acid) formation by *Bacillus licheniformis* in controlled batch fermentor cultures. Biotechnol Bioeng 50:222–227

Cutting SM, Vander Horn PB (1990) Genetic analysis. In: Harwood CR, Cutting SM (eds), Molecular biological methods for *Bacillus*. Wiley, New York, pp 27–74

DeBerry DW, Viehbeck A (1988) Inhibition of pitting corrosion of AISI 304L stainless steel by surface active compounds. Corrosion 44:299–305

Fontana MG (1986) Corrosion engineering, 3rd edn. McGraw-Hill, New York

Hara T, Fujio Y, Seinosuke U (1982) Polyglutamate production by *Bacillus subtilis* (natto). J Appl Biochem 4:112–120

He LM, Neu MP, Vanderberg LA (2000) *Bacillus licheniformis* γ-glutamyl exopolymer: physicochemical characterization and U(VI) interaction. Environ Sci Technol 34:1694–1701

Hefter GT, North NA, Tan SH (1997) Organic corrosion inhibitors in neutral solutions; part 1 – inhibition of steel, copper, and aluminum by straight chain carboxylates. Corrosion 53:657–667

Hernandez G, Kucera V, Thierry D, Pedersen A, Hermansson M (1994) Corrosion inhibition of steel by bacteria. Corrosion 50:603–608

Jayaraman A, Cheng ET, Earthman JC, Wood TK (1997) Axenic aerobic biofilms inhibit corrosion of SAE 1018 steel through oxygen depletion. Appl Microbiol Biotechnol 48:11–17

Jayaraman A, Lee C-C, Chen MW, Mansfeld FB, Wood TK (1999a) Inhibiting sulfate-reducing bacteria in biofilms by expressing the antimicrobial peptides indolicidin and bactenecin. J Ind Microbiol 22:167–175

Jayaraman A, Örnek D, Duarte DA, Lee CC, Mansfeld FB, Wood TK (1999b) Axenic aerobic biofilms inhibit corrosion of copper and aluminum. Appl Microbiol Biotechnol 52:787–790

Ko YH, Gross RA (1998) Effects of glucose and glycerol on γ-poly(glutamic acid) formation by *Bacillus licheniformis* ATCC 9945a. Biotechnol Bioeng 57:430–437

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Mansfeld F (1976) The polarization resistance technique for measuring corrosion currents. In: Fontana MG, Staehle RW (eds) Advances in corrosion science and technology. Plenum Press, New York, pp 163–262

Mansfeld F, Shih H (1988) Detection of pitting with electrochemical impedance spectroscopy. J Electrochem Soc 135:1171–1172

Mansfeld F, Lin S, Kim S, Shih H (1989) Electrochemical impedance spectroscopy as a monitoring tool for passivation and localized corrosion of aluminum alloys. Mater Sci Forum 44–45:83–95

Mansfeld F, Lin S, Shih H (1992a) Detection and monitoring of localized corrosion of aluminum alloys with electrochemical impedance spectroscopy. Am Soc Test Mater Spec Tech Publ 1134:141–152

Mansfeld F, Tsai CH, Shih H (1992b) Software for simulation and analysis of electrochemical impedance spectroscopy (EIS) data. Am Soc Test Mater Spec Tech Publ 1154:186–196

Mansfeld F, Hsu CH, Örnek D, Wood TK, Syrett BC (2000) Corrosion control using regenerative biofilms (CCURB) on aluminum 2024 and brass in different media. In: Mansfeld F, Huet F, Mattos O (eds) New trends in electrochemical impedance spectroscopy (EIS) and electrochemical noise analysis (ENA). The Electrochemical Society PV 2000–24, Pennington, N.J. pp 99–118

McLean RJC, Beauchemin D, Clapham L, Beveridge TJ (1990) Metal-binding characteristics of the gamma-glutamyl capsular polymer of *Bacillus licheniformis* ATCC 9945. Appl Environ Microbiol 56:3671–3677

Mueller E, Sikes CS, Little BJ (1993) Peptide interactions with steel surfaces: inhibition of corrosion and calcium carbonate precipitation. Corrosion 49:829–835

Neidhardt FC, Ingraham JL, Schaechter M (1990) Physiology of the bacterial cell. Sinauer, Sunderland, Mass.

Örnek D, Jayaraman A, Wood TK, Sun Z, Hsu CH, Mansfeld F (2001) Pitting corrosion control using regenerative biofilms on aluminium 2024 in artificial seawater. Corros Sci 43:2121–2133

Rodriguez RL, Tait RC (1983) Recombinant DNA Techniques: An Introduction. The Benjamin/Cummings Publishing Company, Menlo Park, CA

Sekine I, Sanbongi M, Hagiuda H (1992) Corrosion inhibition of mild steel by cationic and anionic polymers in cooling water system. Electrochem Soc 139:3167–3173

Shih H, Mansfeld F (1989) A fitting procedure for impedance spectra obtained for cases of localized corrosion. Corrosion 45:610–614

Smith AW, Iglewski BH (1989) Transformation of *Pseudomonas aeruginosa* by electroporation. Nucleic Acids Res 17:10509

Snyder L, Champness W (1997) Molecular genetics of bacteria. American Society for Microbiology, Washington, D.C.

Sutherland IW (1982) Biosynthesis of microbial exopolysaccharides. In: Rose AH, Morris JG (eds) Advances in microbial physiology, vol 23. Academic Press, London, pp 79–147

Tran LP, Nagai T, Itoh Y (2000) Divergent structure of the ComQXPA quorum-sensing components: molecular basis of strain-specific communication mechanism in *Bacillus subtilis*. Mol Microbiol 37:1159–1171

Vasantha N, Thompson LD, Rhodes C, Banner C, Nagle J, Filpula D (1984) Genes for alkaline protease and neutral protease from *Bacillus amyloliquefaciens* contain a large open reading frame between the region coding for signal sequence and mature protein. J Bacteriol 159:811–819

Vasantha N, Helene A, Mario C, Joachim R (1992) Modular expression and secretion vectors for *Bacillus subtilis*. Gene 114:121–126

Wu X-C, Lee W, Tran L, Wong S-L (1991) Engineering a *Bacillus subtilis* expression-secretion system with a strain deficient in six extracellular proteases. J Bacteriol 173:4952–4958