Letters in Applied Microbiology 2000, **30**, 28–32

Detection of GroEL in activated sludge: a model for detection of system stress

A.J. Duncan, C.B. Bott, K.C. Terlesky and N.G. Love

Virginia Polytechnic Institute and State University, Department of Civil and Environmental Engineering, Blacksburg, VA and the Fralin Biotechnology Center, Blacksburg, VA, USA

2078/99: received 9 January 1999 and accepted 20 August 1999

A.J. DUNCAN, C.B. BOTT, K.C. TERLESKY AND N.G. LOVE. 2000. GroEL is a ubiquitous constitutively synthesized protein that is also stress inducible. Activated sludge, which is a standard biological process used in wastewater treatment systems, is made up of a diverse microbial consortium. The synthesis of GroEL in activated sludge was significantly induced after heat (42 °C) shock. The increased level of GroEL expression was shown to be due to *de novo* protein synthesis. We have demonstrated a method which shows that stress proteins can be detected in activated sludge, and propose their use as specific indicators of system stress.

INTRODUCTION

In wastewater treatment processes such as activated sludge, the removal of biodegradable organic matter is accomplished by a diverse population of micro-organisms through aerobic and/or anaerobic treatment. Contamination of this system by toxic compounds perturbs the treatment process; as a result, removal of biodegradable chemical pollutants from the incoming wastewater can be inhibited (Monteith *et al*. 1995; O'Brien and Teather 1995; Bhattacharya *et al*. 1996; Paxéus 1996). Rapid detection of potentially ineffective wastewater treatment due to system stress from chemical shocks would be beneficial. Current monitoring technologies convey limited information about the physiological status of wastewater treatment system cultures and rarely predict treatment performance problems, resulting in the discharge of improperly treated wastewater (Sanchez *et al*. 1988; Dorn *et al*. 1991).

Current indicators of the 'health' of activated sludge may include pH, dissolved oxygen changes, oxidation-reduction potential and/or oxygen uptake rate (WERF 1994). Except for the latter, these methods are indirect. Use of oxygen uptake rate as an indicator of culture 'health' is of limited use as an increasing number of innovative biological treatment systems incorporate anaerobic treatment zones at the front end of the process. Luminescing biosensors for detection of specific chemicals (Heitzer *et al*. 1994; Rogers and Gerlach 1996) or stress responses (Van Dyk *et al*. 1994; Van Dyk *et al*. 1995; Belkin *et al*. 1997) are being developed. These have the potential to indicate the presence of bioavailable toxins in wastewater. Unfortunately, these sensors are restricted to aerobic environments for online use, since the biochemistry of the luminescing reaction requires molecular oxygen (Burlage 1997) and cannot be used directly in anaerobic mixed liquor samples. Conversely, the induction of stress proteins may serve as the basis for monitoring the status of biotreatment system cultures under a broad range of redox conditions because stress proteins are not limited to expression under aerobic conditions (Spector *et al*. 1986; Terlesky and Tabita 1991). New biosensor technologies, such as antibody-based biosensors, have the potential to provide continuous monitoring in aqueous samples (Rogers and Gerlach 1996). However, before this and similar technologies can be applied to monitor the microbial stress response in complex activated sludge cultures, methods are needed that detect stress proteins in these cultures and studies must be conducted to determine if stress proteins are useful indicators of culture 'health'.

Few researchers have attempted to extract and examine total proteins present in activated sludge samples by SDS–PAGE. Huber *et al*. (1997)) demonstrated different protein expression patterns by two-dimensional gel-electrophoresis in laboratory batch activated sludge reactors fed with different substrates. To date, no field-scale activated sludge studies have focused on short-term changes in protein expression patterns due to sudden changes in wastewater composition or operational practices.

GroEL is a general stress protein which belongs to the family of heat-shock proteins (Hsps). Stress proteins, including GroEL, are rapidly produced in response to different

Correspondence to: Dr Nancy Love, Department of Civil and Environmental Engineering, 418 New Engineering Building, Blacksburg, VA 24061–0246, USA (e-mail: nlove@vt.edu).

types of physical and chemical stresses (VanBogelen *et al.* 1987; Matin 1990; Little *et al.* 1991; Völker *et al.* 1994; Kilstrup *et al.* 1997). Besides general stress proteins, microorganisms frequently respond by inducing specific proteins that are characteristic of the stressor (Blom *et al.* 1992; Lupi *et al.* 1995). The induction of unique, specific stress proteins has been shown in many pure culture experiments (primarily with *Escherichia coli*), although the structure and function of most of these proteins remain uncharacterized. We believe that protein induction patterns (or protein fingerprints) in activated sludge cultures exposed to chemical stressors will provide useful information regarding the potential for treatment process deterioration. Before initiating a study on the generation and diversity of stress proteins useful for wastewater treatment monitoring, we believed it was important to demonstrate that a common stress protein could be induced and detected in complex activated sludge cultures using conventional immunochemical techniques. This paper reports the use of the general stress protein GroEL and its induction in response to heat using environmentally relevant pure cultures as well as complex activated sludge cultures. Our overall goal was to develop a laboratory method for detecting known stress proteins in activated sludge.

MATERIALS AND METHODS

Activated sludge and primary effluent sources

Freshly mixed liquor activated sludge samples were taken from the aerobic basin at the Peppers Ferry Regional Wastewater Treatment Facility, Radford, Virginia. This plant incorporates both organic carbon and ammonia oxidation in a single sludge aerobic process. Primary effluent was obtained from the primary clarifier. The temperature of the field samples was 20 °C. All samples were returned to the laboratory and used immediately.

Bacterial strains and media

All the following bacterial strains were grown on media recommended by the American Type Culture Collection (ATCC), except where noted. *E. coli* strain 7118 was grown in Luria broth (medium 1065), *Rhodobacter sphaeroides* ATCC 17023 was grown in R8AH medium (medium 550), *Nitrosomonas europaea* ATCC 19718 was grown in *Nitrosomonas* medium (medium 221), *Sphingomonas capsulata* ATCC 14666 was grown in nutrient broth (medium 3) and *Pseudomonas putida* ATCC 33015 was grown in nutrient broth (medium 3 – different from ATCC recommended medium), all at 20 °C with shaking at 250 r.p.m. (incubator shaker model G25, New Brunswick Scientific Co. Inc., Edison, NJ, USA).

Antibodies, production and specificity

Antiserum to purified *R. sphaeroides* GroEL (Terlesky and Tabita 1991) was raised in a male New Zealand white rabbit at Cocalico Biologicals (Reamstown, PA, USA). Initial inoculation was performed using 100 μg GroEL antigen mixed with complete Freund's adjuvant. Subsequent boosts with 50 μg GroEL antigen and incomplete Freund's adjuvant were performed on d 14, 21 and 49 post-initial inoculation. The final bleed was performed on d 56 post-initial inoculation and the maximum titre of GroEL antibody in the serum was determined experimentally by Western blotting. No further purification of the antibody was necessary. Pre-immune serum was collected and showed no reaction with GroEL or activated sludge proteins by Western blotting. The reactivity of the antiserum generated was compared to two commercially available antibodies directed at proteins isolated from *E. coli*, one antibody to GroEL and one to DnaK (Stressgen Biotechnologies Corp., BC, Canada). Both these antibodies were monoclonal, and no commercially available polyclonal antisera to these proteins were found.

Sample preparation, electrophoresis and immunodetection

Undiluted activated sludge cultures were mixed by diffused aeration for the duration of each experiment. Experiments were conducted for 1 h at either 20 °C (control) or 42 °C (heat shocked). Pure cultures were also incubated at 20 °C or 42 °C (1 h) with shaking at 250 r.p.m. In one experiment, chloramphenicol was added at 34 μg ml^{-1} (*E. coli*) or 200 μg ml^{-1} (activated sludge) prior to heat shock to confirm *de novo* synthesis of GroEL.

Activated sludge and pure culture cell-free extracts (CFE) were prepared as follows: 1·5 ml samples were pelleted at 15 000 *g* for 5 min, resuspended in 100 μl of cell-free extract buffer [50 mmol/l Tris (pH 7·5), 10 mmol/l EDTA, 10 mmol/l sodium chloride, 0·1 mmol/l PMSF, 5% (v/v) glycerol)] and lysed by sonication [3 × 10 s bursts with 10-s rests on ice using a 3·2-mm diameter microprobe with power output at 40% (Ultrasonic homogenizer, 4710 series, Cole-Parmer Instrument Co., Chicago, IL, USA)]. Insoluble debris was removed by centrifugation (15 000 *g* for 10 min) and the protein content of each CFE was measured using the bicinchoninic acid method as per the manufacturer's instructions (Sigma Chemical Co, St Louis, MO, USA). The CFE was diluted 1 : 4 with electrophoresis sample buffer [0·18 mol/l Tris-HCl (pH 6·8), 15% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0·075% (w/v) bromophenol blue, 0·3% (w/v) (SDS)] and heated to 100 °C for 5 min. After centrifugation (15 000 *g* for 5 min) proteins in the supernatant fluid were separated on 10% denaturing polyacrylamide gels using a Hoefer Mighty Small SE260 mini-gel system according to

the manufacturer's instructions (Pharmacia Biotech, San Francisco, CA, USA). All samples were loaded at equivalent concentrations of protein (15 μg per lane). The antigens were transferred to nitrocellulose membranes (0·45 μm) using a Bio-Rad Mini Transblot cell (Hercules, CA, USA) for 1·5 h at 70 V. The membranes were blocked for 30 min with 5% (w/v) non-fat dry milk in phosphate-buffered saline/0·1% (v/v) TWEEN 20, after which they were incubated with the primary antibody for 1·5 h [1 : 10 000 rabbit anti-GroEL (polyclonal); 1 : 1000 mouse anti-GroEL (Stressgen Biotechnologies Corp., BC, Canada); 1 : 1000 mouse anti-DnaK (Stressgen Biotechnologies Corp., BC, Canada)]. After incubation for 1 h in the appropriate alkaline-phosphatase conjugated secondary antibody [1 : 10 000 goat antirabbit (Sigma, St Louis, MO, USA); 1 : 2000 goat antimouse (Biorad, Hercules, CA, USA)], colour development was achieved using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) according to the manufacturer's instructions (Gibco BRL, Grand Island, NY, USA).

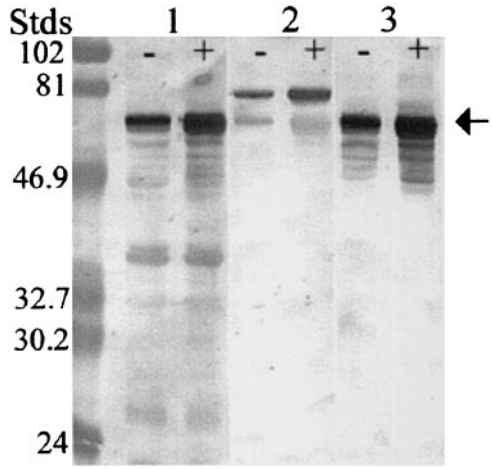

Fig. 1 Immunoblot of *E. coli* strain 7118, control (–) and heat-shocked (+) probed with antiserum against GroEL (lane 1), monoclonal antibody to DnaK (lane 2) and monoclonal antibody to GroEL (lane 3). Stds: molecular weights given in kDa (Bio-Rad, Hercules, CA, USA). Arrow indicates position of GroEL.

RESULTS AND DISCUSSION

The commercial monoclonal antibody directed against GroEL was found to be species-specific, and did not detect the presence of GroEL in activated sludge cultures collected from the field (data not shown). Antiserum for this study that was not species-specific was made. The reactivity of our antiserum to GroEL from *E. coli* was confirmed by comparing it to the monoclonal antibodies to GroEL and DnaK. The position and intensity of the immunoreactive banding pattern was similar for our anti-GroEL antiserum and the monoclonal antibody to GroEL in *E. coli*, but not for the monoclonal to DnaK (Fig. 1).

Figure 2 shows that the antiserum generated reacts well with GroEL from a variety of bacterial genera. The bacteria used in this study are known to be common in activated sludge biotreatment systems (Mobarry *et al.* 1996; Snaidr *et al.* 1998) and represent environmentally relevant strains. This broad reactivity to our GroEL antiserum is an important property for probing activated sludge samples that are made up of a diverse population of micro-organisms.

As shown by SDS–PAGE (Fig. 3a) the induction of a protein presumed to be GroEL (based on the molecular weight) is clearly visible in the heat-shocked culture of *E. coli* (+). The induction of this protein was visually determined to be inhibited in the presence of chloramphenicol. Conversely, activated sludge protein banding patterns are extremely diffuse.

Although SDS–PAGE alone does not allow the identification of newly induced proteins in activated sludge culture extracts, Western blots of activated sludge extracts can be used to identify proteins for which antisera exist. It can be seen using immunodetection with our antiserum that the

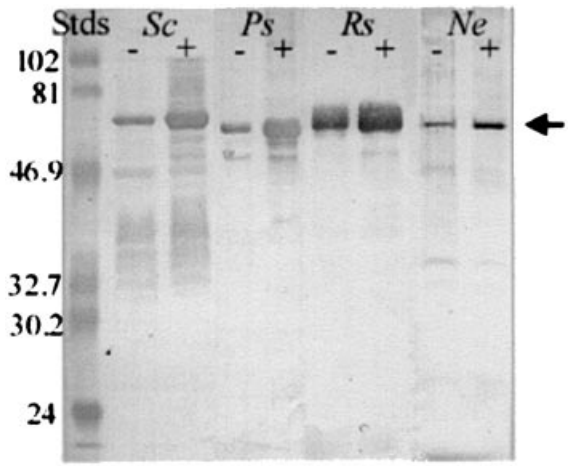

Fig. 2 Immunoblot showing that antiserum raised against GroEL from *Rhodobacter sphaeroides* reacts with GroEL from different bacterial Genera. *Sphingomonas capsulata* ATCC 14666 (*Sc*), *Pseudomonas putida* ATCC 33015 (*Ps*), *Rhodobacter sphaeroides* ATCC 17023 (*Rs*) and *Nitrosomonas europaea* ATCC 19718 (*Ne*) control (–) and heat-shocked (+) cultures probed with antiserum to GroEL. Arrow indicates position of GroEL. Stds: molecular weights given in kDa (Bio-Rad, Hercules, CA, USA).

levels of GroEL in both *E. coli* and activated sludge samples were elevated in heat-shocked samples relative to controls (Fig. 3b). Additionally, the lack of increased induction in the presence of chloramphenicol clearly demonstrates that the elevated levels of GroEL detected in *E. coli* and activated sludge samples exposed to heat shock were due to *de novo* protein synthesis.

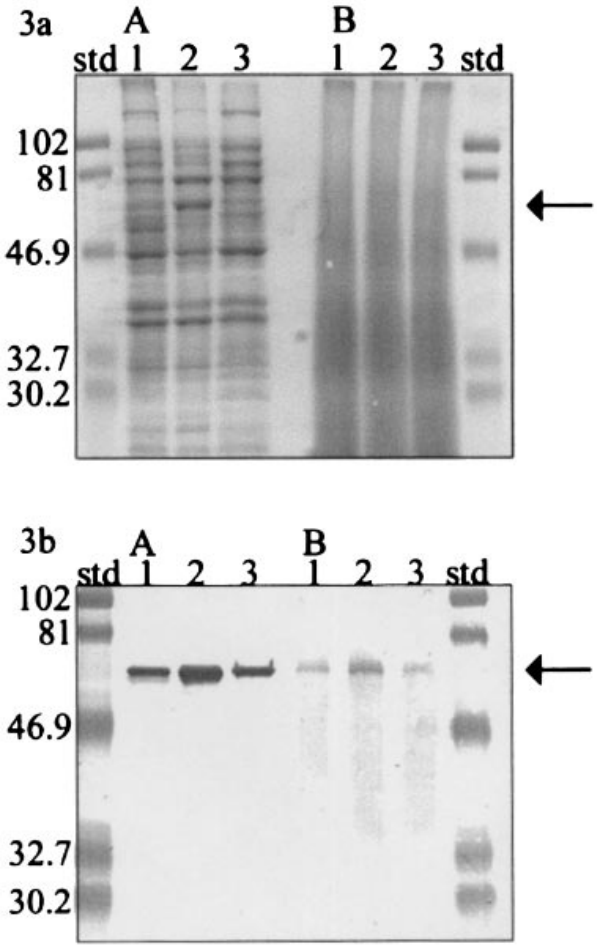

Fig. 3 (a) Coomassie blue stained SDS–PAGE gel and (b)Western blot of both *E. coli* strain 7118 (A), and activated sludge extract (B). Lanes: 1 = control, 2 = heat shock, 3 = heat shock + chloramphenicol. Std: molecular weights given in kDa (Bio-Rad, Hercules, CA, USA). Arrows indicate position of GroEL.

Although GroEL is turned on in response to many different factors, we have shown that it is possible to detect and differentiate between control and heat-stressed samples of activated sludge using conventional immunochemical techniques with a polyclonal antiserum to a known stress protein. We conclude that changes in the levels of GroEL, and probably other as yet undetected stress proteins, in activated sludge provide an indication of rapid changes in the physiological status of the system. These changes are likely to reflect changes in cultures that will lead to treatment process deterioration. We believe that it is *relative* levels of stress proteins in conjunction with their induction patterns that may lead eventually to the development of a useful monitoring technology for activated sludge treatment systems that is based on the microbial stress response. Considering the GroEL data presented here, we believe that it will be possible to use Western blotting to monitor other universal or unique stress proteins in activated sludge cultures exposed to chemical stress conditions. Information on stress protein induction patterns may correlate with wastewater treatment process performance problems so that the mechanism responsible for process deterioration can be better understood.

ACKNOWLEDGEMENTS

This work was supported by the National Science Foundation, Grant No. BES 95–02450. We would like to thank R. Tabita from The Ohio State University for providing an initial sample of antibodies to GroEL from *R. sphaeroides*.

REFERENCES

Belkin, S., Smulski, D.R., Dadon, S., Vollmer, A.C., Van Dyk, T.K. and LaRossa, R.A. (1997) A panel of stress-responsive luminous bacteria for the detection of selected classes of toxicants. *Water Research* **31,** 3009–3016.

Bhattacharya, S.K., Madura, R.L., Dobbs, R.A., Angara, R.V.R. and Tabak, H. (1996) Fate of selected RCRA compounds in a pilot-scale activated sludge system. *Water Environmental Research* **68,** 260–269.

Blom, A., Harder, W. and Matin, A. (1992) Unique and overlapping pollutant stress proteins of *Escherichia coli*. *Applied and Environmental Research* **58,** 331–334.

Burlage, R.S. (1997) Emerging technologies: bioreporters, biosensors, and microprobes. In *Manual of Environmental Microbiology* eds Hurst, C.J., Knudsen, G.R., McInerney, M.J., Stetzenbach, L.D. and Walter, M.V. pp. 115–123. Washington DC: American Society for Microbiology.

Dorn, P.B., van Compernolle, R. and Meyer, C.L. (1991) Aquatic hazard assessment of the toxic fraction from the effluent of a petrochemical plant. *Environmental Toxicology and Chemistry* **10,** 691–703.

Heitzer, A., Malachowsky, K., Thonnard, J.E., Bienkowski, P.R., White, D.C. and Sayler, G.S. (1994) Optical biosensor for environmental on-line monitoring of naphthalene and salicylate bioavailability with an immobilized bioluminescent catabolic reporter bacterium. *Applied and Environmental Microbiology* **60,** 1487–1494.

Huber, S., Minnebusch, S., Wuertz, S., Wilderer, P.A. and Helmreich, B. (1997) Impact of different substrates on protein composition in biomass during wastewater treatment investigated by two-dimensional electrophoresis. In *Proceedings from the Second International Conference on Microorganisms in Activated Sludge and Biofilm Processes* eds Jenkins, D. and Hermanowicz, S.W. pp. 645–648. Berkeley, CA: International Association on Water Quality.

Kilstrup, M., Jacobsen, S., Hammer, K. and Vogensen, F.K. (1997) Induction of heat shock proteins DnaK, GroEL, and GroES by salt stress in *Lactococcus lactis*. *Applied and Environmental Microbiology* **63,** 1826–1837.

Little, C.D., Fraley, C.D., McCann, M.P. and Matin, A. (1991) Use of bacterial stress promoters to induce biodegradation under conditions of environmental stress. In *On-Site Bioreclamation*

Processes for Xenobiotic and Hydrocarbon Treatment eds Hinchee, R.E. and Olfenbuttel, R.F. pp. 493–498. Boston: Butterworth-Heinemann.

Lupi, C.G., Colangelo, T. and Mason, C.A. (1995) Two-dimensional gel electrophoresis analysis of the response of *Pseudomonas putida* KT2442–2-chlorophenol. *Applied and Environmental Microbiology* **61,** 2863–2872.

Matin, A. (1990) Molecular analysis of the starvation stress in *Escherichia coli*. *FEMS Microbial Ecology* **74,** 185–196.

Mobarry, B.D., Wagner, M., Urbain, V., Rittmann, B.E. and Stahl, D. (1996) Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. *Applied and Environmental Microbiology* **62,** 2156–2162.

Monteith, H.D., Parker, W.J., Bell, J.P. and Melcer, H. (1995) Modeling the fate of pesticides in municipal wastewater treatment. *Water Environmental Research* **67,** 964–970.

O'Brien, G.J. and Teather, E.W. (1995) A dynamic model for predicting effluent concentrations of organic priority pollutants from an industrial wastewater treatment plant. *Water Environmental Research* **67,** 935–942.

Paxéus, N. (1996) Organic pollutants in the effluents of large wastewater treatment plants in Sweden. *Water Research* **30,** 1115–1122.

Rogers, K.R. and Gerlach, C.L. (1996) Environmental biosensors: a status report. *Environmental Science and Technology* **30,** 486a–491a.

Sanchez, P.S., Sato, M.I.Z., Paschoal, C.M.R.B., Alves, M.N. and Furlan, E.V. (1988) Toxicity assessment of industrial effluents from S. Paulo State, Brazil, using short-term microbial assays. *Toxicity Assessment* **3,** 55–80.

Snaidr, J., Amann, R., Huber, I., Ludwig, W. and Schleifer, K.-H. (1998) Phylogenetic analysis and identification of bacteria in activated sludge. *Applied and Environmental Microbiology* **63,** 2884–2896.

Spector, M.P., Aliabadi, Z., Gonzalea, T. and Foster, J.W. (1986) Global control I in *Salmonella typhimurium*: two-dimensional electrophoretic analysis of starvation-, anaerobiosis-, and heat shock-inducible proteins. *Journal of Bacteriology* **168,** 420–424.

Terlesky, K.C. and Tabita, F.R. (1991) Purification and characterization of the chaperonin 10 and chaperonin 60 proteins from *Rhodobacter sphaeroides*. *Biochemistry* **30,** 8181–8186.

VanBogelen, R.A., Kelley, P.M. and Neidhardt, F.C. (1987) Differential induction of heat shock, SOS, and oxidation stress regulons and accumulation of nucleotides in *Escherichia coli*. *Journal of Bacteriology* **169,** 26–32.

Van Dyk, T.K., Majarian, W.R., Konstantinov, K.B., Young, R.M., Dhurjati, P.S. and LaRossa, R.M. (1994) Rapid and sensitive pollutant detection by induction of heat shock gene-bioluminescence gene fusions. *Applied and Environmental Microbiology* **60,** 1414–1420.

Van Dyk, T.K., Reed, T.R., Vollmer, A.C. and LaRossa, R.A. (1995) Synergistic induction of the heat shock response in *Escherichia coli* by simultaneous treatment with chemical inducers. *Journal of Bacteriology* **177,** 6001–6004.

Völker, U., Engelmann, S., Maul, B. *et al.* (1994) Analysis of the induction of general stress proteins in *Bacillus subtilis*. *Microbiology* **140,** 741–752.

Water Environment Research Foundation (WERF) (1994) *On-line monitoring to control transients in wastewater treatment*. Water Environment Project 92-OPW-1, Alexandria, VA.