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Overcoming Drug Resistance with Alginate Oligosaccharides Able To Potentiate the Action of Selected Antibiotics

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The uncontrolled, often inappropriate use of antibiotics has resulted in the increasing prevalence of antibiotic-resistant pathogens, with major cost implications for both United States and European health care systems. We describe the utilization of a low-molecular-weight oligosaccharide nanomedicine (OligoG), based on the biopolymer alginate, which is able to perturb multidrug-resistant (MDR) bacteria by modulating biofilm formation and persistence and reducing resistance to antibiotic treatment, as evident using conventional and robotic MIC screening and microscopic analyses of biofilm structure. OligoG increased (up to 512-fold) the efficacy of conventional antibiotics against important MDR pathogens, including *Pseudomonas*, *Acinetobacter*, and *Burkholderia* spp., appearing to be effective with several classes of antibiotic (i.e., macrolides, β-lactams, and tetracyclines). Using confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM), increasing concentrations (2%, 6%, and 10%) of alginate oligomer were shown to have a direct effect on the quality of the biofilms produced and on the health of the cells within that biofilm. Biofilm growth was visibly weakened in the presence of 10% OligoG, as seen by decreased biomass and increased intercellular spaces, with the bacterial cells themselves becoming distorted and uneven due to apparently damaged cell membranes. This report demonstrates the feasibility of reducing the tolerance of wound biofilms to antibiotics with the use of specific alginate preparations.

The evolution of multidrug resistance (MDR) represents an increasingly formidable challenge in the developed and developing worlds, having a colossal clinical and economic impact (26). This escalating problem is a major world-health issue, compounded by the lack of new antibiotics in development, particularly for Gram-negative MDR bacteria. The need for new strategies to improve the diagnosis and treatment of these infections is, therefore, acute (15, 16).

In vivo, the inability to eradicate bacteria from a diverse number of clinical situations ranging from the periodontal pocket to the cystic fibrosis (CF) lung has been attributed to the acquisition of a biofilm state (12, 13). Antimicrobial resistance can also arise by a plethora of mechanisms involving chromosomal mutations or horizontal gene transfer. Resistance may result from destruction of the antibiotic by, e.g., β-lactamases, typified by the recently described New-Delhi metallo-β-lactamase-1 (NDM-1) (21); mutations in specific antibiotic target sites, e.g., RNA polymerase and DNA gyrase (22); limitation of local antibiotic concentrations due to outer membrane lipid bilayer modifications or porin-mediated permeability (5); the action of specific multidrug efflux pump mechanisms, e.g., MexAB-OrpM (28); acquisition of resistance genes such as *mecA* in methicillin-resistant *Staphylococcus* aureus (MRSA) (22); or the sequestration of antibiotics within the bacterial biofilm (25).

Attempts to develop (and screen) alternative antibacterial strategies to potentiate the activity of conventional antibiotics are becoming increasingly important. Antibacterial strategies have included use of antimicrobial cationic peptides (AMPs) (7); cell membrane permeabilization (7); molecular chaperones (e.g., heat shock proteins) (4); inhibitors of RecA-mediated strand exchange (34); inhibitors of DNA synthesis (6); inhibitors of penicillin-binding protein production (18); and use of efflux pump inhibitors or inhibitors of beta-lactamase expression (27). Despite almost 20 years of intense research, none of these agents deemed useful *in vitro* have progressed to clinical use.

Alginate is a biopolymer which is found in brown algae and bacterial extracellular polysaccharide (EPS) and is a linear polymer composed of (1–4)-linked α-L-guluronate (G) and β-D-mannuronate (M) residues. It is extensively utilized in foods and medicines (e.g., wound dressings) due to its physical cross-linking and gel-forming properties (30). Sodium alginate is recognized as safe by the U.S. Food and Drug Administration (reference no. 21CFR184.1724). While naturally occurring alginate is polydispersed in relation to molecular weight and G/M composition, it is possible to generate polymers of defined molecular weight and also G/M composition (11), which have been employed to modulate immunological responses *in vivo* (35). Previous workers have highlighted the potential of nanoscale biosensor-based diagnostics for the detection of life-threatening bacterial infections (16). We describe here the development of the first polymer therapeutics (based on alginate oligosaccharides) designed to specifically modulate bacterial responses and treat infections of MDR bacteria, with phase IIa human clinical studies under way.

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TABLE 1 Strains used for susceptibility testing and their known relevant genotypes[a]

Strain no.	Strain designation	Relevant genotype(s)	Origin
V1	*Pseudomonas aeruginosa* (R22)	VIM-2, *aac61b*, *aadA7*[b,c]	China
V2	*Pseudomonas aeruginosa* (301)	VIM-2[b,c]	Poland
V13	*Pseudomonas aeruginosa* (PAO1, ATCC 15692)		Australia
V79	*Pseudomonas aeruginosa* (ATCC 39324)		United States
V33	*Burkholderia cepacia* (ATCC 25416)	—[c]	United States
V51	*Burkholderia contaminans* (LMG 23255)	—[c]	Czech Republic
V52	*Burkholderia cenocepacia* (AU1054)	—[c]	United States
	Burkholderia dolosa (LMG 18941)		United States
V19	*Acinetobacter baumannii*	—[b,c]	United Kingdom
V9	*Acinetobacter baumannii*	VIM-2[b,c]	Egypt
V4	*Acinetobacter baumannii*	—[b,c]	Libya
V22	*Acinetobacter lwoffii*	NDM-1[b,c]	United Kingdom
V10	*Acinetobacter lwoffii*	VIM-2[b,c]	Tunisia
V3	*Klebsiella pneumoniae*	NDM-1[b,c]	India
V6	*Klebsiella pneumoniae*	NDM-1[b,c]	India
V5	*Escherichia coli*	AIM-1[b,c]	Australia
V7	*Escherichia coli*	NDM-1[b,c]	India
U204	*Staphylococcus aureus* NCIMB 9518		United States
E68	*Staphylococcus aureus* NCTC 6571		United Kingdom
U50	*Staphylococcus aureus* 1040 (MRSA)	*mecA*	United Kingdom
V17	*Streptococcus oralis* 5610		United Kingdom

[a] AIM-1, metallo-β-lactamase; NDM-1, New Delhi metallo-β-lactamase; MRSA, methicillin-resistant *Staphylococcus aureus*.
[b] Sensitive to colistin only.
[c] MDR, multidrug resistant (resistant to 3 or more classes of antibiotic).

MATERIALS AND METHODS

Bacterial strains. The strains used for susceptibility testing are shown in Table 1. They include both culture collection strains and clinical isolates: *Pseudomonas aeruginosa* ($n = 4$), *Burkholderia* spp. ($n = 4$), *Acinetobacter* spp. ($n = 4$), *Enterobacteriaceae* spp. ($n = 4$), *Staphylococcus aureus* ($n = 3$), and *Streptococcus oralis* ($n = 1$). These isolates represent the most frequently encountered resistance types (aminoglycosides, carbapenems, cephalosporins, fluoroquinolones, monobactams, and penicillins).

Bacterial media and culture conditions. Bacterial colonies were grown on blood agar base no. 2 (BA; LabM) supplemented with 5% horse blood and tryptone soya broth (TSB; Oxoid) for liquid cultures. Biofilms were generated in cation-adjusted Mueller-Hinton (MH) broth (LabM). Biofilms were washed with phosphate-buffered saline (PBS). All antibiotics (Sigma-Aldrich, Gillingham, United Kingdom, or Bristol-Myers Squibb Pharmaceuticals Ltd.) used were pharmaceutical grade and included the major classes of antibiotics (β-lactams, aminoglycosides, macrolides, tetracyclines, carbapenems, and polymyxins) employed in the treatment of these organisms. Ultrapurified pig gastric mucin glycoprotein (purified by Jeff Pearson, Newcastle University) and OligoG (2,600 Da, 90 to 95% G) were provided by AlgiPharma AS, Sandvika, Norway.

Synthesis of alginate oligosaccharides. OligoG was generated from alginate extracted from the stem of the locally sourced brown seaweed *Laminaria hyperborea*. Purification and fractionation of the resulting material yielded an oligomer with a high content of guluronate and a relatively narrow molecular-weight distribution. Final purification using charcoal filters was followed by spray drying. The test OligoG had 90% to 95% of the monomer residues as G residues (mean, 2,600 M_w; Fig. 1A). Purified OligoG was characterized by hydrogen-1 nuclear magnetic resonance (H-NMR) (Fig. 1B) and high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD; Fig. 1C). OligoG is anionic and highly soluble, although its solubility is limited by viscosity at higher concentrations (>15%), the rise in viscosity being offset to a degree by an increase in temperature. OligoG was tested at working concentrations of 2% to 10%, phase 1 studies having demonstrated that these concentrations are safe for clinical use (clinical phase 1 safety and toxicology studies; www.clinicaltrials.gov [identifier, NCT00970346]).

Alginate oligosaccharides of similar molecular weights but altered M content were generated via epimerisation technology and also tested. M-rich alginates (pure mannuronan) from a nonpathogenic high-expressing mutant (*P. fluorescens* NCIMB10525) were used as a substrate for mannuronan C-5-epimerase AlgE4, converting alternating M residues on the alginate backbone into G residues. These were developed with similar molecular-weight profiles and defined, based on G:M composition, as OligoM (100% M; 3,000 to 3,500 M_w) (11) and OligoMG (46% G; 4,000 to 4,500 M_w) (3).

Effects of OligoG on bacterial growth in liquid culture. The effects of alginate oligosaccharide OligoG on a collection of clinically relevant human Gram-negative and Gram-positive MDR and non-MDR pathogens (Table 1) were studied using robotic high-throughput screening (HTS; Beckman-Coulter) and conventional screening and a range (0% to 10%) of OligoG concentrations. Overnight (O/N) bacterial cultures grown in tryptone soya broth (TSB) were inoculated into Mueller-Hinton (MH) broth with increasing concentrations (0%, 2%, 6%, and 10%) of OligoG in microtiter plates. After cultivation for 19 h, cell densities were determined by measuring optical density at 600 nm (OD_{600}). The OligoG-induced reduction in optical density observed in these assays correlates directly with reduced bacterial dry weight/biomass (data not shown).

Growth of biofilms for SEM imaging or LIVE/DEAD staining. The ability of OligoG to modulate the development of biofilms as well as their effect on 24-h biofilms *in vitro* was investigated. Overnight cultures of *P. aeruginosa* PAO1 were grown in TSB. After dilution of the bacterial cultures to 0.5 McFarland in MH broth with mucin (2.5 g/liter), 1 ml was transferred to the wells of a flat-bottom 12-well plate containing sterile plastic Thermanox coverslips (Agar Scientific, Essex, United Kingdom) for scanning electron microscopy (SEM) or glass coverslips (Fisher Scientific) for confocal laser scanning microscopy (CLSM). Plates were then wrapped in parafilm to prevent dehydration and incubated at 37°C for 6 h to allow biofilm formation. Planktonic cells and supernatant were removed and biofilms washed with 1 ml sterile PBS. Cells then were treated with a combination of OligoG (0%, 2%, 6%, and 10%) and mucin (2.5 g/liter) in 1 ml MH broth. Plates were then wrapped in parafilm and incubated at 37°C for 24 h with gentle agitation.

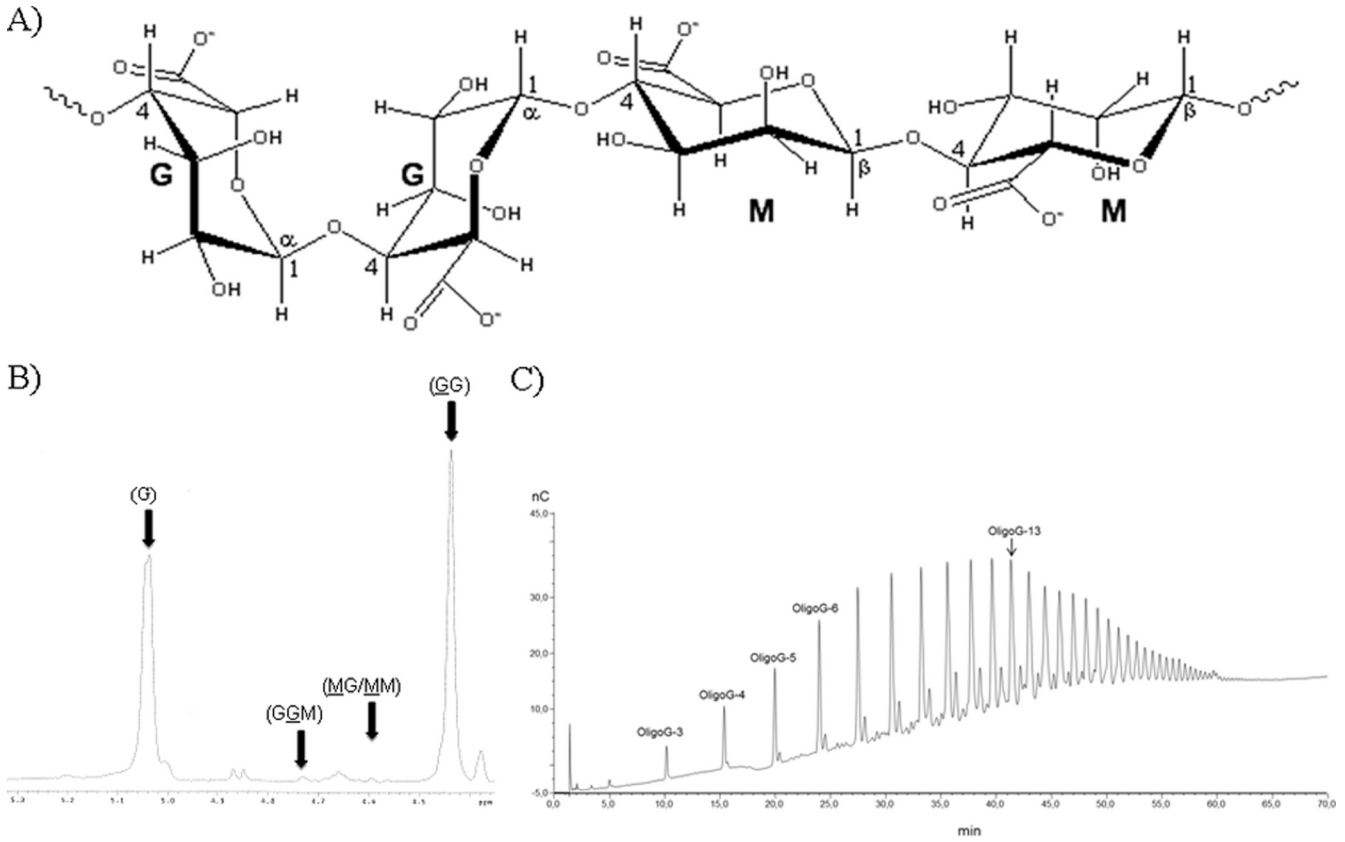

FIG 1 Structure, purification, and quality control of OligoG. (A) Structures of α-L-guluronate (G) and β-D-manuronate (M); OligoG has at least 90% to 95% of the monomer residues as G residues. (B) NMR spectrum of OligoG containing 96% G. (C) Characterization of OligoG with high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

SEM of OligoG-treated *Pseudomonas* biofilms. Glutaraldehyde was added to OligoG-treated biofilms at a final concentration of 1.25% and fixed at room temperature for 24 h. The samples were dehydrated in a graded series of ethanol concentrations, dried in a critical point dryer (CPD 030; Balzers, Germany), mounted on aluminum stubs, coated with gold in a sputter-coater (model AE 1231; EMscope, United Kingdom), and then viewed on a scanning electron microscope (XL-20; FEI-Philips, The Netherlands).

LIVE/DEAD staining and CLSM of OligoG-treated biofilms. The effect of OligoG on cell membrane integrity (a measure of cell death) was investigated using LIVE/DEAD *Bac*Light stain (bacterial viability kit; Invitrogen). A 2-μl volume of each stain (green fluorescent SYTO9 and red-fluorescent propidium iodide) was added to 1 ml NaCl (0.85% [wt/vol]) and mixed and 100 μl added to each test sample. The preparation was incubated in the dark for 15 min and then analyzed by CLSM using a Leica TCS SP2 AOBS spectral confocal microscope (Leica, Wetzlar, Germany).

OligoG-treated gfp-labeled *Pseudomonas aeruginosa* PAO1 biofilms and staining of biofilm matrix for CLSM. A gfp (green fluorescent protein)-labeled *P. aeruginosa* PAO1 derivative (20) was also used for CLSM. Overnight cultures of gfp-PAO1 were grown in TSB, and 10 μl was used to inoculate 1 ml MH broth with or without 2%, 6%, or 10% OligoG. Then, 200 μl of each was transferred to individual Coverwell incubation chamber gaskets (Invitrogen) with the addition of 40 μl BODIPY 630/650-X SE stain (Invitrogen), which stains extracellular polysaccharide (EPS) matrix components. The chamber gaskets were then sealed with a Histobond (R. A. Lamb Ltd., Eastbourne, United Kingdom) positively charged slide. Each unit was inverted and incubated for 18 h at 37°C to allow biofilm formation on the Histobond slide. Coverwell incubation chamber gaskets and any supernatant and planktonic cells were removed from the Histobond slide and replaced with a glass coverslip. Undisturbed biofilms were then visualized by CLSM as described above.

Susceptibility testing by MIC assay. Potentiation of alginate oligomers was studied using standard MICs (19) and confirmed by robotic HTS using a range of international MDR (i.e., resistant to 3 or more classes of antibiotics) and non-MDR Gram-negative and Gram-positive clinical isolates (Table 1). Isolates were grown overnight in TSB and then diluted in PBS until the OD_{625} was between 0.08 and 0.10 (equivalent to 0.5 McFarland standard; approximately 10^8 CFU/ml). Two-fold antibiotic serial dilutions were prepared in MH broth or MH broth with OligoG at 2%, 6%, or 10% in flat-bottom 96-well microtiter plates (100 μl in each well). The diluted bacterial cultures were diluted 10-fold in MH broth, and 5 μl was added to the microtiter plates containing the antibiotic serial dilutions to give a final concentration of 5×10^5 CFU/ml. Plates were incubated at 37°C for 16 to 20 h and MICs determined as the lowest concentration at which there was no visible growth. As well as conventional culture, a robotic high-throughput screening system (see below) was used to determine MICs.

Robotic HTS. Serial dilutions for MIC determinations were performed with a Tecan Genesis RSP 200 liquid-handling workstation equipped with an 8-channel pipetting tool using sterile disposable 200-μl barrier tips. The optical density of the cultures was measured using a Beckman Coulter Robotic Core system with an integrated Beckman Coulter Biomek NX^P robotic liquid-handling unit, a Thermo Cytomat 2 450 S robotic incubator, and a Beckman Coulter Paradigm microplate reader. The 384-well microplates were incubated at 37°C for 19 h. The microplates were shaken at 1,800 rpm (2.5-mm amplitude) for 120 s prior to taking absorbance readings at 600 nm. HTS was used for determining

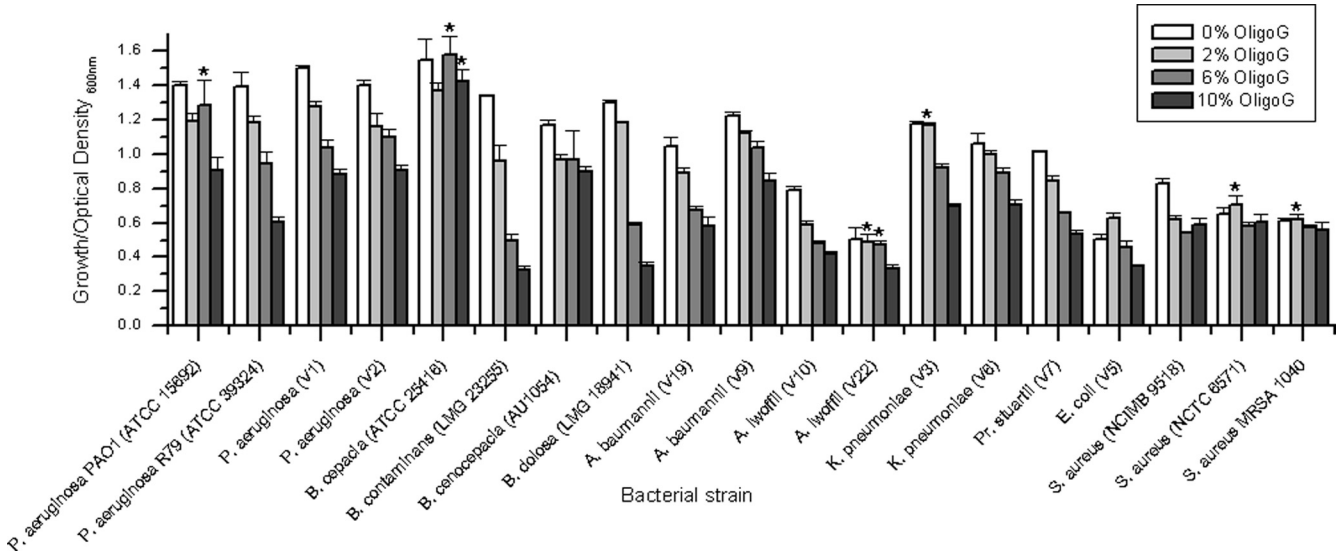

FIG 2 Effect of increasing concentrations of OligoG (0%, 2%, 6%, and 10%) on cell densities after cultivation for 19 h for various Gram-negative bacterial species. (*, data not significantly different from control results; $P > 0.05$).

the growth curves obtained at various concentrations of OligoG as well as for confirming the results from the conventional MIC testing.

Effect of pulmonary surfactant on antimicrobial efficacy of OligoGs. Previous studies have shown that surfactants in the lung can interact with antibiotics, for example, with daptomycin (33), resulting in an inhibition of antibacterial activity. Here, beractant (Survanta) (a natural bovine lung extract) was used to mimic lung surfactant to determine whether it interfered with the ability of the OligoG to enhance antibiotic antimicrobial activity. In these experiments, 10% beractant was incorporated into the MIC test with OligoG.

When beractant was added to MH broth, a white precipitate formed and persisted, even after incubation of the MIC plate at 37°C overnight. It was unclear if this apparent cloudiness was due to the beractant precipitating or to bacterial growth. As this obscured reading of the plates, to determine the MIC values for beractant, it was necessary to subculture 5 μl from each well of the MIC plate into fresh MH broth with overnight incubation at 37°C prior to determining the correct cutoff points between regrowth and clear wells.

Effect of OligoG on MexAB-OrpM efflux pump mutants. MIC assays were carried out as described above with or without OligoG using efflux pump *P. aeruginosa* PAO1 mutant strains alongside the wild-type strain. These included strain K1169, a *mexB* knockout lacking MexAB-OprM, and a *nalB* hyperexpression mutant of MexAB-OprM (10).

Effect of OligoG on bacterial motility. Two methods of motility testing were used. For the plate assay, overnight (O/N) cultures of *Proteus mirabilis* (NSM6) and *Pseudomonas aeruginosa* (PAO1) were grown in TSB at 37°C. Cultures were diluted 1:100 in MH broth supplemented with 0%, 0.2%, 0.5%, 2%, 6%, or 10% OligoG and reincubated for 18 h at 37°C. Iso-sensitest (ISO) agar plates (Oxoid) containing 0%, 0.5%, 2%, or 6% OligoG were prepared and inoculated with 10 μl of the MH cultures. (ISO plates with 10% OligoG were not used, as they did not set at that concentration.) Plates were incubated for 23 h at 37°C and values for distance of bacterial spread recorded at 2, 5, 7, 13, and 23 h.

For the stab assay, overnight cultures of *P. mirabilis* (NSM6) and *P. aeruginosa* PAO1 and control isolates of *P. mirabilis* (NCTC 11938), *Staphylococcus aureus* (NCTC 6571), *P. aeruginosa* (NCTC 10662), and *Escherichia coli* (NCTC 10418) were prepared in MH broth with or without OligoG as described above. Motility Test Agar (MTA; Mast Group Ltd., Bootle, United Kingdom) was supplemented with 0%, 0.2%, 0.5%, 2%, and 6% OligoG, with 5 ml pipetted into sterile bijou tubes. (MTA containing 10% OligoG also did not set.) The MTA was stab inoculated with the prepared MH broth cultures and incubated for 24 h at 37°C. Motility appeared as a progressive lateral diffuse pink/red color in the MTA and was scored from 0 to 4 as previously described (2). Absence of growth beyond the track of inoculation was scored as 0, and growth throughout the medium was scored as 4.

Testing development of resistance to OligoG. Daily subculturing of *P. aeruginosa* PAO1 in MH broth or MH broth with 10% OligoG was carried out over a 16-day period (>160 generations). Serial passage in escalating concentrations (0.1%, 1%, and 10%) of OligoG was also undertaken ($n = 7$ days for each concentration). MIC assays of the control and test cultures (on day 16 and day 21, respectively) were then performed to see if there were any apparent differences.

RESULTS

Effects of OligoG on growth of MDR bacteria. OligoG was found to reduce bacterial proliferation in liquid culture at 19 h (Fig. 2). This effect was dose dependent for the concentration range investigated (0%, 2%, 6%, and 10%), showing a general decrease in percent change (compared to the control) with increasing OligoG concentration. These effects were particularly striking for the Gram-negative pathogens *P. aeruginosa*, *A. baumannii* and *Burkholderia* spp., and *Enterobactericeae*.

Effects of OligoG on biofilms generated by MDR Gram-negative bacteria examined using conventional light, scanning electron, and confocal laser scanning microscopy. SEM, LIVE/DEAD BacLight staining of PAO1 biofilms, and CLSM of gfp-labeled PAO1 biofilms generated in the presence of OligoG revealed dose-related changes (Fig. 3). Increasing concentrations (0% to 10%) of OligoG resulted in disruption of established 24-h biofilms (demonstrable by cell damage and disruption of cellular content as visualized by SEM), increased cell death (LIVE/DEAD staining), and decreased cellular density (CLSM) in the developed biofilm. CLSM showed that OligoG was able to considerably reduce gfp-labeled PAO1 biofilm formation, with clear dose-dependent alterations in the 3-dimensional structural organization of cells in the biofilm. These changes were also associated with in-

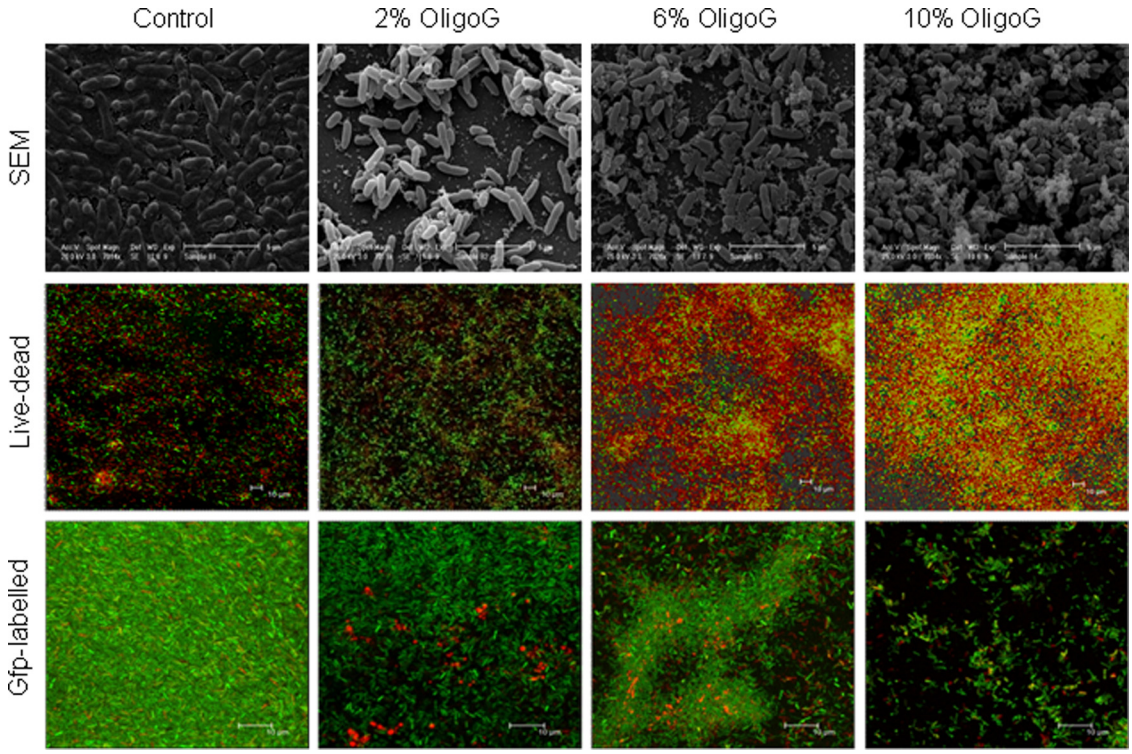

FIG 3 Growth of 24-h *Pseudomonas aeruginosa* PAO1 biofilms in 0%, 2%, 6%, or 10% OligoG visualized by scanning electron microscopy (SEM) or confocal laser scanning microscopy (CSLM) of LIVE/DEAD staining (green, live; red, dead) or CSLM of green fluorescent protein (Gfp)-labeled PAO1 with counterstaining using BODIPY 630/650-X SE extracellular polysaccharide dye.

creasing numbers of nonviable bacteria seen by LIVE/DEAD staining.

The ability of alginate oligomers to potentiate the effect of existing antibiotics on Gram-negative MDR pathogens. The ability of alginate oligomers to potentiate the activity of the principal classes of antibiotics commonly employed in clinical practice in the treatment of Gram-negative infections (cephalosporins, macrolides, quinolones, monobactams, tetracyclines, and carbapenems) was assessed, with selected results for three antibiotic classes shown in Table 2.

OligoG treatment reduced (by up to 512-fold) the MICs of a range of antibiotics, including macrolides (azithromycin, erythromycin, spiromycin, and clarithromycin), aztreonam, ceftazidime, oxytetracycline, and ciprofloxacin. For example, *P. aeruginosa* V2 showed a ceftazidime MIC reduction from 16 to 0.5 μg/ml, representing a change from untreatable (>8 μg/ml) (9) to treatable (<8 μg/ml) levels (0% and 10% OligoG, respectively; Table 2). This potentiation effect was particularly marked for the genus *Burkholderia*. Interestingly, while this potentiation was not seen in the control OligoMG oligomer, a similar, although smaller, effect (up to 32-fold reduction for *P. aeruginosa* and *A. baumannii*; data not shown) was evident in part for OligoM. Specific antibiotic combination effects (using two antibiotics) were also particularly evident (data not shown); for example, using *A. baumannii* and ceftazidime or ciprofloxacin with azithromycin at 1 μg/ml and 10% OligoG resulted in a reduction in the MIC of these combination antibiotics to <1 μg/ml or <0.25 μg/ml, respectively (representing a >512-fold reduction). Similar potentiation, although a lesser effect, was observed with Gram-positive isolates (Table 2). OligoG-antibiotic synergy was not evident for every combination studied. Moreover, no potentiation was seen for the aminoglycoside (tobramycin and amikacin) or the polymyxin (colistin) antibiotics for any bacteria tested.

Antimicrobial susceptibility testing, performed in the presence and absence of artificial surfactant (to mimic the lung environment and investigate any potential nonspecific inhibition which may occur *in vivo*), showed no apparent effect of OligoG at any tested concentration (0% to 10%; data not shown).

Mechanistic cellular and molecular studies to determine the mode of action of OligoG. The addition of OligoG appeared to modulate cellular structure. However, this did not appear to correlate with increased bacterial cell membrane permeability, as the effect contrasted with that observed with an EDTA control (data not shown). Longitudinal studies employing *P. aeruginosa* PAO1 MexAB-OrpM mutants also demonstrated that the effect of OligoG was not mediated via interaction with this efflux pump system, as no differences in MIC determinations with OligoG were noted using wild-type strains or knockout or hyperexpression mutants of MexAB-OrpM, showing that OligoG was not a substrate of MexAB-OrpM (data not shown). Interestingly, motility test agar stabs showed that OligoG (6%) was able to almost completely inhibit motility in *P. aeruginosa*, *E. coli*, and *P. mirabilis* (Fig. 4A). Furthermore, swarming motility in *Proteus* sp. was inhibited in a dose-dependent manner with increasing concentrations of OligoG (0% to 6%), but the effect was predominant in the presence of OligoG (Fig. 4B). Importantly, when the bacteria were exposed to OligoG only in the initial liquid growth medium (and not in the agar plate onto which they were then inoculated), the

effect of OligoG was almost, but not quite, completely lost, showing a slight "carryover" of effect after exposure to OligoG was removed (Fig. 4C).

Resistance to OligoG. MIC determinations of *P. aeruginosa* PAO1 grown in OligoG for prolonged serial subculture and in escalating concentrations of OligoG showed that there was no difference between the test (with OligoG) and control results, indicating no buildup of resistance to OligoG in this time period (data not shown).

DISCUSSION

The functional properties of alginates are strongly correlated with their M/G ratio and with their uronic acid sequence (31). Initial experiments showed that treatment with OligoG impaired bacterial growth. These observed effects were bacteriostatic and may be related to the ability of alginates to chelate cations, particularly iron (32). G subunits are known to show preferential binding to cations such as Ca^{2+}, although not all cations appear to utilize the G subunits as preferred binding sites (24). The magnitude of the antimicrobial effect was considerably potentiated using these low-molecular-weight OligoG oligomers. This inhibition was evident in the studies on bacterial biofilms, where OligoG not only inhibited biofilm formation and resulted in disruption of established 24-h biofilms but also potentiated antibiotic treatment. This observation was at first counterintuitive, as alginate *per se* is an important component of the EPS of certain bacterial biofilms and therapies targeting alginate, e.g., alginate lyases are currently being developed as antibiofilm therapies (23). However, unlike alginates derived from algae, bacterial alginates from most bacteria, such as *P. aeruginosa*, differ considerably structurally, having an absence of G blocks (homopolymeric regions of poly-L-guluronate) (31), which may be a contributing factor in these observed antimicrobial effects.

Structural analysis of the biofilms indicated that OligoG treatment was associated with increased cellular "clumping" and disruption of cellular contents. Hence, the mechanism by which OligoG exerts its effect could simply have been the result of permeabilization of the bacterial membrane, which is the mode of action of a number of antibiotics. However, permeabilization does not appear to be responsible for the cellular membrane changes induced by OligoG treatment, since they contrasted markedly with those observed with permeabilizing agents (EDTA; data not shown). This membrane effect, independent of permeability changes, was reassuring, as, while a number of "permeabilizing" antimicrobial agents have been developed, progress to clinical utility has been hampered by formulation difficulties and nonspecific permeabilization-toxicity concerns (14). The lack of permeabilization reflected the bacteriostatic activity of the oligomers observed in the HTS growth assay. OligoG treatment (both alone and in combination with antibiotics) clearly altered the bacterial membrane. Membrane disruption in AMPs, via the ionic displacement of divalent cations (Mg^{2+} and Ca^{2+}), has been previously described and is a recognized mechanism by which the action of antibiotics may be potentiated (8). While many antimicrobials act on the biosynthetic pathways of growing cells, the bacterial membrane represents an important target in the treatment of quiescent nonreplicating bacteria in recalcitrant infection such as the CF lung (17). A number of agents have been developed that modulate changes in the bacterial membrane (via alterations in NADH2 and ATP synthase and, indirectly, via gen-

TABLE 2 MICs of antibiotics alone and with increasing concentrations (2%, 6%, and 10%) of OligoG for a range of MDR clinical isolates

Isolate	Antibiotic MIC (μg/ml) at indicated OligoG concn (%)[a]																			
	AZM				ERY				Oxy-TET				ATM				CAZ			
	0	2	6	10	0	2	6	10	0	2	6	10	0	2	6	10	0	2	6	10
P. aeruginosa PAO1	128	64	32	8	128	64	64	16	8	4	4	4	8	8	4	2	2	2	2	2
P. aeruginosa V1	128	32	8	4	128	64	32	2	8	4	2	1	8	8	4	2	2	2	2	2
P. aeruginosa V2	8	4	2	0.5	256	128	32	8	1,024	1,024	1,024	1,024	32	16	8	4	16	8	2	0.5
B. cepacia ATCC 25416	32	16	4	0.125	64	64	16	8	128	64	16	2	256	8	2	2	64	8	2	0.25
B. contaminans LMG 23255	2	2	1	0.5	64	32	16	8	32	16	8	2	128	32	<4	<4	8	4	0.25	0.25
B. cenocepacia AU1054	16	8	4	4	128	64	32	32	64	32	16	16	1,024	1,024	128	<4	64	8	8	0.25
A. baumannii V19	32	8	1	<0.25	16	8	2	<0.5	>256	>256	>256	>256	64	32	16	4	128	64	32	32
A. baumannii V9	16	4	0.5	<0.25	8	4	1	<0.5	2	2	1	1	>512	512	256	128	>512	512	512	256
A. lwoffii V10	<0.25	<0.25	<0.25	<0.25	<0.5	<0.5	<0.5	<0.5	0.5	0.5	0.5	0.25	32	16	4	1	2	2	<0.5	<0.5
K. pneumoniae V3	128	64	32	32	1,024	1,024	512	256	8	8	8	4	4,094	2,048	1,024	512	4,096	2,048	2,048	1,024
K. pneumoniae V6	512	256	64	32	256	128	128	2	2	2	4	2	4,096	2,048	512	256	4,096	4,096	2,048	2,048
E. coli V7	8	4	1	0.5	32	16	8	4	4	4	4	2	1,024	512	256	64	>4,096	>4,096	>4,096	>4,096
S. aureus NCIMB 9518	0.5	0.25	0.125	0.031	0.25	0.25	0.063	<0.03	<0.25	<0.25	<0.25	<0.25	512	512	512	512	8	8	2	1
S. aureus NCTC 6571	0.25	0.25	0.125	<0.03	0.25	>16	0.063	<0.03	<0.25	<0.25	<0.25	<0.25	512	256	256	128	4	8	4	2
S. aureus MRSA 1040	512	256	256	256	>1,024	>1,024	1,024	512	2	2	1	0.5	1,024	1,024	512	512	>16	16	8	2
S. oralis 5610	8	2	<0.25	<0.25	2	16	0.031	0.063	<0.25	<0.25	0.5	<0.25	<0.5	<0.5	<0.5	<0.5	8	4	0.031	1

[a] AZM, azithromycin; ATM, aztreonam; CAZ, ceftazidime; ERY, erythromycin; oxy-TET, oxytetracycline. Shaded area represents potentiation of antibiotic with increasing OligoG concentration.

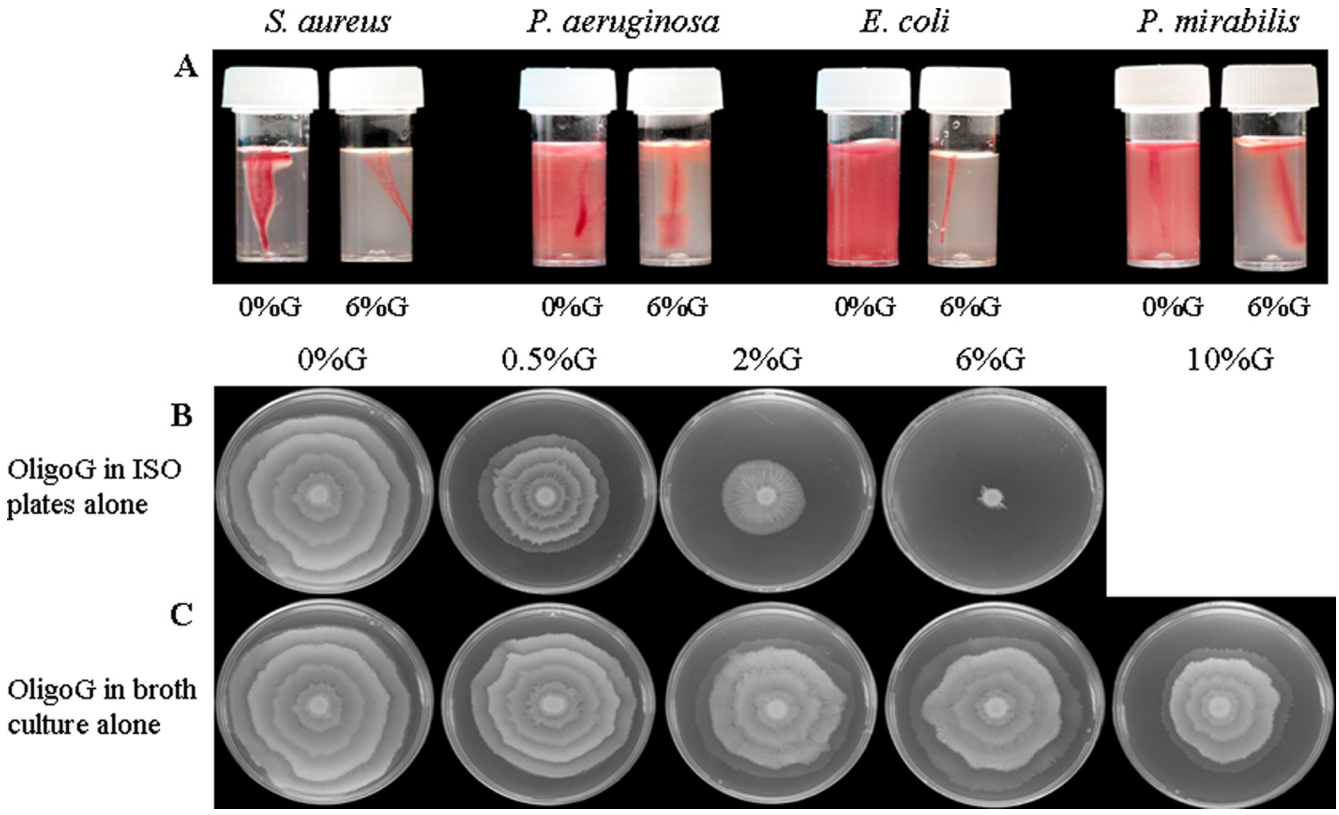

FIG 4 Motility testing. (A) Motility test agar with or without OligoG inoculated with *S. aureus* (negative control; NCTC 6571), *P. aeruginosa* (PAO1), *E. coli* (NCTC 10418), or *P. mirabilis* (NSM6). (B) Broth cultures of *P. mirabilis* (NSM6) grown in MH broth (no OligoG) and plated on ISO agar containing 0%, 0.5%, 2%, or 6% OligoG. (C) Broth cultures of *P. mirabilis* (NSM6) grown in MH broth with 0%, 0.5%, 2%, 6%, or 10% OligoG and plated on ISO agar (no OligoG).

eration of lethal reactive oxygen species and nitric oxide in the bacterial membrane). The observation of the putative, but as-yet-unidentified, membrane effect seen with OligoG is supported by the observed failure to develop resistance during prolonged treatment in longitudinal experiments. Weakened swarming motility has been shown to lead to an impaired ability to form simple biofilms (29). The impairment of bacterial motility by OligoG may, at least in part, explain its mode of action in motile bacteria. The finding that OligoG exhibits activity against a number of nonmotile bacterial species indicates, however, that other mechanisms are undoubtedly involved.

The potentiation of antibiotic therapy in the treatment of MDR organisms in persistent infections is a major goal of anti-infective therapy (17). Indeed, synergistic combined therapies appear to have increasing potential for future antibacterial therapies (1). In characterizing the ability of OligoG to potentiate the activity of conventional antibiotics against MDR organisms, we utilized conventional microbiological MIC assays. These experiments demonstrated that exposure to OligoG was able to decrease the MIC values of Gram-negative pathogens up to 512-fold in a range of MDR organisms tested. Moreover, the MIC levels were significantly reduced for a number of important Gram-negative and, to a lesser extent, Gram-positive pathogens (including MRSA). Furthermore, OligoG proved effective against a variety of multi- and pan-drug-resistant pathogens for which there are currently no or only limited treatments available and hence are often associated with mortality in infected individuals or which represent potential agents of bioterrorism (e.g., *Burkholderia* spp.). EUCAST MIC breakpoint tables (11) describe the use of azithromycin for the treatment of *Salmonella enterica* serovar Typhi infections with an MIC of ≤16 mg/liter (for wild-type isolates). In this study, other *Enterobacteriacae* (encoding NDM-1) such as *Escherichia coli* (V7) showed an 8-fold MIC decrease for azithromycin, indicative that potentiation with OligoG had moved the MIC from >16 μg/ml in the resistance category (i.e., above the breakpoint) to ≤16 μg/ml in the sensitive category (i.e., below the breakpoint). Synergistic activity for organisms such as *Burkholderia* spp. is encouraging, since *Burkholderia* infection is associated with a fatality rate of almost 50% in infected cystic fibrosis patients. Overall, OligoG provides a major advancement in therapeutic development for the treatment of Gram-negative infections such as those caused by *Burkholderia* and *Acinetobacter* spp. for which effective treatment is currently extremely limited.

OligoG has been screened and proven for safety and tolerability in a series of animal and human experiments. Due to their physicochemical properties, alginates are widely employed in the food and pharmaceutical industry, and the lack of toxicity of inhaled (nebulized) OligoG in the animal and human phase 1 studies was, therefore, unsurprising (data not shown). While a number of *in vitro* antibiotic-potentiating agents have been cited in the literature, *in vivo* many have problems with potential safety and toxicity issues. The advantage of OligoG is that it is effectively inert and resists enzymatic degradation. The toxicity and pK studies

demonstrate that the putative doses at which OligoG is effective *in vitro* are safely attainable in the lung *in vivo*.

OligoG represents a novel target nanomedicine for future clinical use. Its suitability for formulation in isotonic solution and safety-pharmacokinetic profiles all favor human use. Furthermore, phase IIa human clinical studies in CF patients are now ongoing.

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