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Polymyxin B Identified as an Inhibitor of Alternative NADH Dehydrogenase and Malate: Quinone Oxidoreductase from the Gram-positive Bacterium *Mycobacterium smegmatis*

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Tuberculosis is the leading cause of death due to a single infectious agent in the world and the emergence of multidrug-resistant strains prompted us to develop new drugs with novel targets and mechanism. Here, we screened a natural antibiotics library with *Mycobacterium smegmatis* membrane-bound dehydrogenases and identified polymyxin B (cationic decapeptide) and nanaomycin A (naphtoquinone derivative) as inhibitors of alternative NADH dehydrogenase [50% inhibitory concentration (IC_{50}) values of 1.6 and 31 μg/ml, respectively] and malate: quinone oxidoreductase (IC_{50} values of 4.2 and 49 μg/ml, respectively). Kinetic analysis on inhibition by polymyxin B showed that the primary site of action was the quinone-binding site. Because of the similarity in K_m value for ubiquinone-1 and inhibitor sensitivity, we examined amino acid sequences of actinobacterial enzymes and found possible binding sites for L-malate and quinones. Proposed mechanisms of polymyxin B and nanaomycin A for the bacteriocidal activity were the destruction of bacterial membranes and production of reactive oxygen species, respectively, while this study revealed their inhibitory activity on bacterial membrane-bound dehydrogenases. Screening of the library with bacterial respiratory enzymes resulted in unprecedented findings, so we are hoping that continuing efforts could identify lead compounds for new drugs targeting to mycobacterial respiratory enzymes.

Key words: *Mycobacterium tuberculosis*, NADH dehydrogenase, natural antibiotics, polymyxin B, respiratory chain.

Abbreviations: IC_{50}, the 50% inhibitory concentration; MDR, multidrug-resistant; MIC, minimum inhibitory concentration; MQO, malate: quinone oxidoreductase; NDH2, alternative NADH dehydrogenase; NQR, NADH: quinone reductase; Q_1, ubiquinone-1; SDH, succinate dehydrogenase.

Tuberculosis is the leading cause of death due to a single infectious agent in the world. Approximately 2 billion people or one-third of the world's population are infected with *Mycobacterium tuberculosis* (*1*). There are 9 million new active cases of tuberculosis per year and ~2 million death (*2*). The emergence of *M. tuberculosis* strains resistant to one or more of the standard first-line agents like isoniazid and rifampicin is a serious concern (*2*, *3*). Multidrug-resistant (MDR) *M. tuberculosis* is difficult to treat, thus, there is an urgent need for discovery and identification of new classes of targets and new antimycobacterial drugs with novel mechanisms of action (*4*, *5*).

Most individuals infected with *M. tuberculosis* are latent carriers who have ~10% risk of developing reactivation of tuberculosis during their life (*6*). *Mycobacterium tuberculosis* is capable of establishing persistent infection in the host by using a complex interplay between host immune system and bacterial survival mechanisms. In hypoxic non-replicating *M. tuberculosis* cells (*e.g.* bacteria in granulomatous or necrotic lesions), the intracellular ATP level was 5–6 times lower than that of aerobic replicating cells, making them exquisitely sensitive to any further depletion, with a 3- to 4-fold ATP depletion resulting >90% cell death (*7*). In the hypoxic respiratory chain, alternative NADH dehydrogenase (NDH2) is essential to replenish the [NAD^+] pool and to energize the cytoplasmic membrane to produce ATP (*7*) and fumarate is used as the terminal electron acceptor (*8*). Both ATP synthase and NDH2 (NdhA) are essential for the viability of *M. smegmatis* and *M. tuberculosis* (*9–12*).

For the aerobic respiratory chain, *M. tuberculosis* and *M. smegmatis* genomes encode genes for three NADH dehydrogenases [H^+-translocating NADH dehydrogenase (NDH1, Complex I) and two NDH2s (NdhA and Ndh) in *M. tuberculosis*, and NdhA (GenBank accession no. YP_887924) and NdhB (YP_888708) in *M. smegmatis*], malate: quinone oxidoreductase (MQO), succinate dehydrogenase (SDH, Complex II), quinol: cytochrome *c* reductase (cytochrome bc_1c, Complex III) and two-terminal oxidases [cytochrome *c* oxidase (Complex IV)

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and cytochrome *bd*-type quinol oxidase]. Through menaquinone, electrons are transferred from dehydrogenases to cytochrome *bd* or to a cytochrome bc_1c/cytochrome *c* oxidase supercomplex (*5*, *13*). A role of NDH1 NuoG in the anti-apoptosis mechanism in infected host cells was recently reported for *M. tuberculosis* (*14*), but the expression of NDH1 in *M. tuberculosis* and *M. smegmatis* remains uncertain (*10*, *11*, *14*, *15*). In *M. leprae*, the whole *nuo* (NDH1) operon is deleted except for a *nuoN* pseudogene (*5*). Sequence identity of NuoG in virulent mycobacterial species and the vaccine strain BCG was 99%, but *M. smegmatis* NuoG is only 70% identical (*14*).

Because of the absence in mammalian mitochondria, NDH2, MQO and terminal quinol oxidases are potential targets for new chemotherapeutics. Recently, we screened natural antibiotics in the Kitasato Institute for Life Sciences Chemical Library (*16*) with bacterial and parasitic protist respiratory enzymes and identified gramicidin S, LL-Z1272β and LL-Z1272ε as inhibitors of *Escherichia coli bd*-type quinol oxidase, LL-Z1272γ, LL-Z1272δ and LL-Z1272ζ as inhibitors of *E. coli* bo_3-type quinol oxidase and trypanosome alternative oxidase (*17*, *18*), gramicidin S and scopafungin as inhibitors of *Gluconobacter oxydans* NDH2 (*19*) and LL-Z1272 γ as an inhibitor of *G. oxydans* cyanide-insensitive oxidase (*20*). From these screening studies, we found that the inhibitory activity of antibiotics varied from species to species. Here, we carried out screening of the natural antibiotics library with *M. smegmatis* membrane-bound dehydrogenases and identified polymyxin B (cationic decapeptide) and nanaomycin A (naphthoquinone derivative) as inhibitors of NDH2 and MQO.

EXPERIMENTAL PROCEDURES

Preparation of M. smegmatis Membranes—*Mycobacterium smegmatis* mc^2 155 cells were grown for 7 days at 37°C in 7H9 broth (Difco) supplemented with 0.05% Tween 80 and ADC (albumin, glucose and catalase) at 100 r.p.m. Stationary-phase cells were collected by centrifugation at 3,500 r.p.m. for 30 min at 4°C and washed twice with 30 mM Tris–HCl containing 10 mM Na-EDTA (pH 7.5). Cells were resuspended in the same buffer containing bacterial protease inhibitor cocktail (Sigma, St. Louis, MO) and disrupted by passage through a French press cell at 13–14 MPa. The lysate was centrifuged at $12{,}000 \times g$ for 15 min at 4°C to remove unbroken cells. Membranes were recovered from the supernatant by centrifugation at $150{,}000 \times g$ for 60 min at 4°C and resuspended in 30 mM Tris–HCl (pH 7.5).

Screening of a Natural Antibiotics Library—Screening of a natural antibiotics library (*16*) with *M. smegmatis* membranes (30–50 μg protein/ml) was carried out at 10 μg/ml in 50 mM Tris–HCl (pH 7.4) containing 0.05% Tween 20 (Calbiochem, San Diego, CA) and 5 mM KCN. For screening with NADH: quinone reductase (NQR), reactions were started by adding 100 μl of the membrane suspension to 100 μl of the reaction mixture containing 20 μg/ml of the natural compounds, 0.2 mM ubiquinone-1 (Q_1) and 0.4 mM NADH in UV-transparent 96-well microplates. For screening with malate: quinone reductase (MQR), 0.4 mM NADH was replaced with 20 mM L-malate. The decrease of the oxidized form of Q_1 was determined with SpectraMax Plus^{384} high-throughput spectrophotometer (Molecular Devices, Sunnyvale, CA) by monitoring the absorbance at 275 nm before and after 10-min incubation at 25°C. Reproducibility was confirmed with the independent preparations. Screening with rat liver mitochondria (*21*) was carried out in 50 mM Tris–HCl (pH 7.4) containing 1 mM $MgCl_2$ and 2 mM KCN.

Measurement of Dehydrogenase Activities—Enzyme assay was performed at 25°C with V-650 spectrophotometer (JASCO, Tokyo, Japan), and reactions were started by addition of substrates (electron donors). NQR activity of the membranes was measured in 50 mM Tris–HCl (pH 7.4) containing 200 μM NADH ($\varepsilon_{340} = 6.22$/mM/cm) and 100 μM Q_1 in the presence of 5 mM KCN. MQR activity was measured by replacing 200 μM NADH with 10 mM L-malate. For inhibition studies, the reaction mixture was preincubated with inhibitors for 2 min. Determinations of the 50% inhibitory concentration (IC_{50}) were done with KaleidaGraph ver. 4.0 (Synergy Software, Reading, PA) using the equation, relative residual activity $= A/(1 + ([IC_{50}/[\text{Inhibitor}])^n)$ where A = total amplitude (≈ 1) and n = Hill coefficient (*17*). Kinetic analysis was carried out as described previously (*17–20*).

RESULTS

Identification of Inhibitors for M. smegmatis Membrane-bound Dehydrogenases—Screening of 304 microbial compounds in the natural antibiotics library (*16*) was carried out at final concentrations of 10 μg/ml and we identified polymyxin B and nanaomycin A as NQR inhibitors, which can reduce the residual activity to <50% (Table 1). *Mycobacterium smegmatis* genome encodes three NADH dehydrogenases, NDH1 and two NDH2 (NdhA and NdhB). In contrast to NDH1, NDH2 cannot oxidize deamino-NADH (reduced nicotinamide hypoxantine dinucleotide) (*22*). Since *M. smegmatis* membranes did not show significant deamino-NADH oxidase activity (<2 mU/mg protein), the NQR activity of the membranes is attributable to NDH2. The divergence in the amino acid sequence of NdhB from those

Table 1. **Effects of natural antibiotics on *M. smegmatis* and rat liver mitochondria membrane-bound dehydrogenases.**

Antibiotics	Residual activity (%)			
	Mycobacterium smegmatis membranes		Rat liver mitochondria	
	NDH2	MQO	NDH1	SDH
Control	100	100	100	100
Polymyxin B	14 ± 2	42 ± 1	79 ± 1	90 ± 4
Nanaomycin A	34 ± 5	43 ± 5	74 ± 18	72 ± 6
Gramicidin S	103 ± 6	82 ± 5	95 ± 3	100 ± 1
Scopafungin	100 ± 2	100 ± 1	100 ± 3	97 ± 4

Amounts of Q_1 reduced (MQO and SDH) or NADH oxidized (NDH1 or NDH2) for 10 min at 25°C were determined at 10 μg/ml antibiotics by 10-min end point assay with a plate reader. Relative residual activity was calculated by dividing the activity with the inhibitor by the control activity.

of *M. smegmatis* NdhA and *M. tuberculosis* NdhA and Ndh, which have been shown to be both active (*5*, *23*), suggests that NdhA is a likely active NADH dehydrogenase species in our membrane preparation. Parallel screening revealed the inhibitory activity of both antibiotics on the MQR activity of MQO (Table 1). No other antibiotics reduced the quinone reductase activity of NDH2 and MQO to <50% of the control activity. Further, the screening with rat liver mitochondria suggests that both polymyxin B and nanaomycin A are less toxic to mammalian NDH1 and succinate dehydrogenase (Table 1).

Determination of IC_{50} values of NDH2 and MQO—In the presence of 0.1 mM Q_1, we examined effects of polymyxin B and nanaomycin A on the initial velocity of the NQR and MQR activities of *M. smegmatis* membranes. IC_{50} values of NDH2 for polymyxin B and nanaomycin A were determined to be 1.6 and 31 μg/ml, respectively (Fig. 1), and IC_{50} of MQO were 4.2 and 49 μg/ml, respectively (Fig. 2). It should be noted that polymyxin E (colisitin), a polymyxin B analogue in our library (Fig. 3), showed a very low-inhibitory activity on NDH2 (IC_{50} = 2.6 mg/ml) and had no effect on MQO (Fig. 2). These results indicate that polymyxin B is a novel inhibitor of *M. smegmatis* NDH2 (IC_{50} of 1.4 μM) and MQO (3.7 μM).

Kinetic Analysis of Inhibition of M. smegmatis NDH2 by Polymyxin B—As we reported recently (*17*, *19*), the screening of the natural antibiotics library can identify new respiratory enzyme inhibitors with no structural similarity to substrates. Both gramicidin S (cationic cyclic decapeptide) and scopafungin (36-membered ring macrolide) are unrelated to quinones, but they inhibited the Q_1-dependent NQR activity of *G. oxydans* NDH2 by competitively (IC_{50} = 1.2 μM) and mixed-type mechanism (6.2 μM), respectively, indicating the proximity of the inhibitor-binding site to the quinone-binding site (*19*).

We carried out kinetic analysis on *M. smegmatis* NDH2 and found that K_m value for Q_1 (15 μM) (Fig. 4) was comparable with 16 μM of *G. oxydans* NDH2 (*19*), but higher than 6 μM of *M. tuberculosis* NDH2 for Q_2 (*23*) and of *E. coli* NDH2 for Q_1 (*24*). In the presence of polymyxin B, enzyme kinetics changed from the Michaelis–Menten type to substrate inhibition kinetics with the substrate inhibition constant K_{si} of 57.4 ± 4.5 μg/ml (Fig. 4). Data for low concentrations of

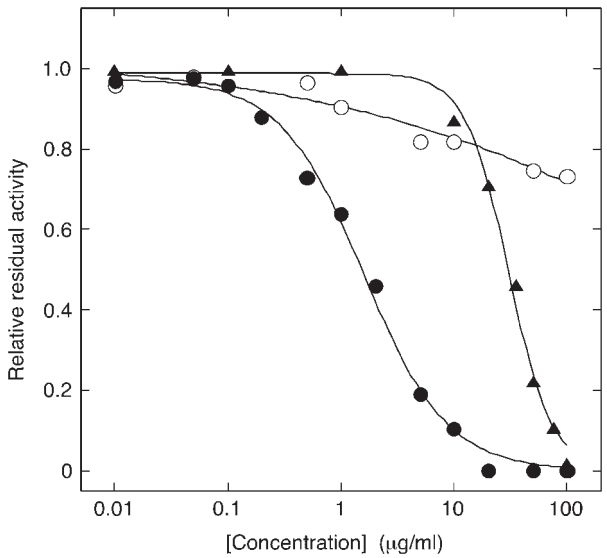

Fig. 1. **Inhibition of *M. smegmatis* NDH2 by polymyxin B, polymyxin E and nanaomycin A.** Effects of polymyxin B (filled circle), polymyxin E (open circle) and nanaomycin A (filled triangle) on the initial velocity of Q_1 reductase activity of NDH2 were examined with V-650 spectrophotometer in the presence of 0.1 mM Q_1 and 0.2 mM NADH. IC_{50} values of NDH2 were estimated to be 1.6 ± 0.1 μg/ml (polymyxin B, n = 1.2 ± 0.1), 2.6 ± 0.05 mg/ml (polymyxin E, n = 0.26 ± 0.09) and 30.6 ± 1.4 μg/ml (nanaomycin A, n = 2.3 ± 0.2).

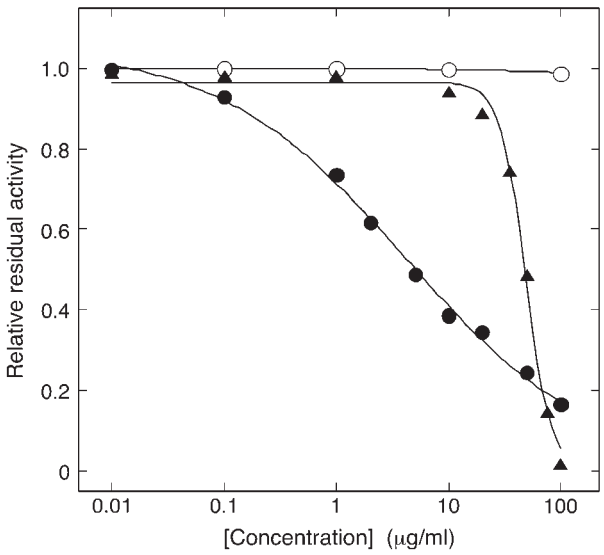

Fig. 2. **Inhibition of *M. smegmatis* MQO by polymyxin B, polymyxin E and nanaomycin A.** Effects of polymyxin B (filled circle), polymyxin E (open cirlce) and nanaomycin A (filled triangle) on the initial velocity of Q_1 reductase activity of MQO were examined with V-650 spectrophotometer in the presence of 0.1 mM Q_1 and 10 mM L-malate. IC_{50} values of NDH2 were estimated to be 4.2 ± 0.6 μg/ml (polymyxin B, n = 0.52 ± 0.03) and 48.9 ± 1.4 μg/ml (nanaomycin A, n = 3.9 ± 0.4).

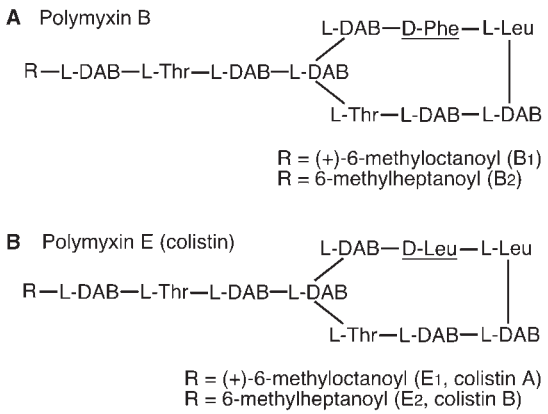

Fig. 3. **Structures of polymyxins B and E.** L-DAB indicates L-α,γ-diaminobutyric acid and amino acids at position 6 were underlined.

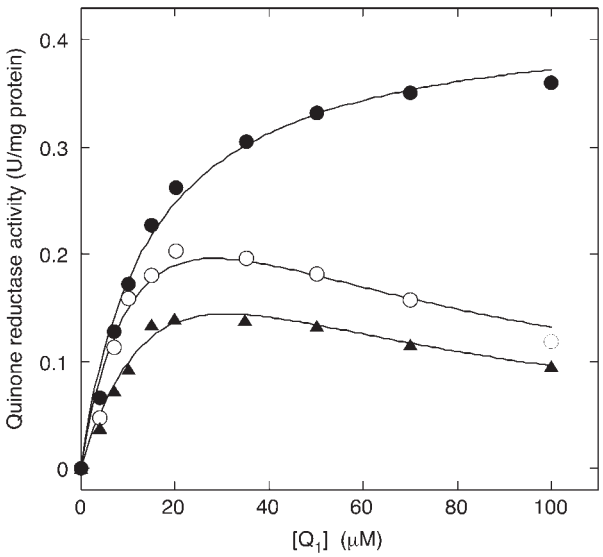

Fig. 4. **Kinetic analysis of inhibition of *M. smegmatis* NDH2 by polymyxin B.** NADH oxidase activity was determined as a function of the Q_1 concentration in the absence (filled circle) or presence of 1 (open circle) and 3 (filled triangle) μg/ml polymyxin B. After 2-min incubation with the inhibitor, reactions were started by addition of 0.2 mM NADH. Data points were the average values from duplicate assay. Data for the control were fitted to the Michaelis–Menten kinetics and the apparent K_m and V_{max} values were estimated to be 14.6 ± 1.4 μM (Q_1) and 0.43 ± 0.01 U/mg protein, respectively. Data in presence of polymyxin B were fitted to the substrate inhibition kinetics and the apparent K_m, V_{max} and K_{si} (*i.e.* constant for substrate inhibition) values were estimated to be 12.2 ± 9.8 μM, 1.16 ± 0.78 U/mg protein and 61.9 ± 48.8 μM (1 μg/ml polymyxin B); and 17.5 ± 10.5 μM, 0.66 ± 0.31 U/mg protein and 52.8 ± 30.4 μM (3 μg/ml polymyxin B). Data for low concentrations of substrate (0–20 μM) were fitted to competitive inhibition kinetics with the K_i value of 2.9 ± 0.8 μg/ml at 1 μg/ml polymyxin B and 2.1 ± 0.2 μg/ml at 3 μg/ml polymyxin B.

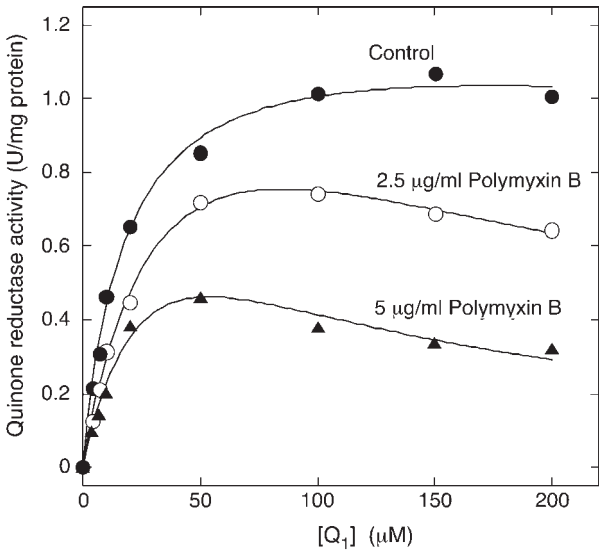

Fig. 5. **Kinetic analysis of inhibition of *M. smegmatis* MQO by polymyxin B.** Quinone reductase activity was determined as a function of the Q_1 concentration in the absence (filled circle) or presence of 2.5 (open circle) and 5 (filled triangle) μg/ml polymyxin B. After 2-min incubation with the inhibitor, reactions were started by addition of L-malate. Data points were the average values from duplicate assay. Data were fitted to the substrate inhibition kinetics and the apparent K_m, V_{max} and K_s values were estimated to be 19.1 ± 2.8 μM (Q_1), 1.26 ± 0.09 U/mg protein and $1{,}600 \pm 1{,}200$ μM (control); 40.1 ± 5.7 μM, 1.46 ± 0.13 U/mg protein and 181 ± 35 μM (2.5 μg/ml polymyxin B); and 40.4 ± 17.1 μM, 1.15 ± 0.34 U/mg protein and 73 ± 34 μM (5 μg/ml polymyxin B). Data for low concentrations of substrate (0–50 μM) were fitted to competitive inhibition kinetics with the K_i value of 2.9 ± 0.2 μg/ml at 2.5 μg/ml polymyxin B and 2.1 ± 0.4 μg/ml at 5 μg/ml polymyxin B.

substrate (0–20 μM) were fitted by competitive inhibition kinetics with the K_i value of 2.5 ± 0.4 μg/ml, being consistent with the possible location of the polymyxin B-binding site near the quinone-binding site. Because of a high IC_{50} value and a high UV-vis absorption, we did not study the inhibitory mechanism of nanaomycin A.

Kinetic Analysis of Inhibition of M. smegmatis MQO by Polymyxin B—Inhibitory mechanism of polymyxin B for MQO was examined as functions of the substrate concentrations. In the absence of polymyxin B, the malate- and Q_1-dependent quinone reduction followed the Michaelis–Menten kinetics. Apparent K_m (L-malate) and V_{max} values for L-malate-dependent reactions were determined to be 2.6 ± 0.1 mM and 1.63 ± 0.01 U/mg protein, respectively, at 0.1 mM Q_1 (data not shown). K_m for L-malate was comparable with 1.2 mM of *E. coli* MQO (*25*). As reported for the inhibition of the NADH-dependent reaction of *G. oxydans* NDH2 by gramicidin S and scopafungin (*19*), polymyxin B inhibited the malate-dependent reaction non-competitively with the K_i value of 7.0 ± 3.5 μg/ml (6 μM) (data not shown).

The Q_1-dependent reaction followed substrate inhibition kinetics, and apparent K_m (Q_1), V_{max} and K_{si} values were estimated to be 19.1 μM, 1.3 U/mg protein and 1.6 mM, respectively, in the absence of polymyxin B at 10 mM L-malate (Fig. 5). K_m for Q_1 was comparable with K_m values of *M. smegmatis* (*23*) and *G. oxydans* NDH2 (*19*). Data for low concentrations of substrate (0–50 μM) were fitted to competitive inhibition kinetics with the K_i value of 2.5 ± 0.4 μg/ml polymyxin B, being consistent with the possible location of the polymyxin B-binding site near the quinone-binding site. These kinetic studies suggest the similarity in the quinone- and polymyxin B-binding sites of NDH2 and MQO.

DISCUSSION

Polymyxins—Polymyxin B and polymyxin E (Fig. 3) are old class of cationic peptide antibiotics and active against MDR Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Enterobacter* species (*26*, *27*). The shortage of new antibiotics to combat against MDR strains has led to the revival of polymyxins for salvage therapy (*27*, *28*). Old reports described the high incidence of nephrotoxicity and neurotoxicity for polymyxin therapy (*29*), and they were replaced in the 1970s by antibiotics considered to be less toxic. However, randomized

controlled trials, *in vitro* pharmacodynamics, animal or clinical studies on pharmacokinetic properties, the development of improved formulations, the optimization of dosing and the evaluation of toxicity are scarce (*30*). Recent studies concluded that polymyxins have acceptable effectiveness and considerably less toxicity than was reported in old studies (*27*).

Polymyxins are pentabasic decapeptide antibiotics containing a cycloheptapeptide ring with a C9 or C10 fatty acid chain [6-methyl-octanoic acid (polymyxin B_1, Polymyxin E_1); 6-methyl-heptanoic acid (polymyxin B_2, polymyxin E_2)] through an α-amide linkage (*31*) (Fig. 3) and non-ribosomally synthesized in *Bacillus polymyxa*. The target of antimicrobial activity is assumed to be the bacterial membrane. Cationic polypeptides bind to anionic lipopolysaccharide molecules in the outer membrane of the Gram-negative bacteria, leading to a local disturbance of the membrane which then causes an increase in the permeability (*32*, *33*). By random screening of the natural antibiotics library, we identified polymyxin B as a novel inhibitor of *M. smegmatis* NDH2 and MQO. We have shown recently that gramicidin S (cationic cyclic decapeptide) serves as a potent inhibitor of *E. coli bd*-type quinol oxidase and *G. oxydans* NDH2 (*17*, *19*), although its primary mode of action is to perturb the lipid bilayer, resulting the destruction of the membrane integrity. Kinetic analyses indicate that these cationic cyclic peptides interact with the quinone-binding site of membrane-bound respiratory enzymes. Although polymyxin B was a potent inhibitor of *M. smegmatis* NDH2, bacteriocidal activity ($IC_{50} = 21$ μg/ml) was weaker than polymyxin E (8 μg/ml) (data not shown). Minimum inhibitory concentration (MIC) of polymyxin E has been reported to be 5 μg/ml (*M. tuberculosis* and *M. avium*) and 30 μg/ml (*M. smegmatis*) (*34*, *35*), while that of polymyxin B was >500 (*M. avium*) and 32 μg/ml (*M. smegmatis*) (*36*). It should be noted that between polymyxins B and E there is only one amino acid difference at position 6 (Fig. 3), which determines the inhibitory activity on NDH2 and MQO and the bacteriocidal activity.

Nanaomycin A—Nanaomycin A has been isolated from *Streptomyces rosa* var. *notoensis* and is active against mycoplasma, fungi and Gram-positive bacteria including *M. smegmatis* (*37*, *38*). MIC of *M. smegmatis* has been reported to be 62.5 μg/ml (*37*). Since nanaomycins are 1,4-naphthoquinone derivatives, the primary mode of action is likely the competition of the quinone-binding site of membrane-bound enzymes with substrates. In the Gram-negative bacterium *Vibrio alginolyticus*, Hayashi *et al*. (*39*) have reported that nanaomycins A and D generated superoxide radicals upon reduction by membrane-bound dehydrogenases including NDH2 (K_m, 20 μM). In *M. smegmatis* membranes, we found no or insignificant evidence for NADH-dependent reduction of nanaomycin A (<1 mU/mg protein at 40 μg/ml nanaomycin A) and production of reactive oxygen species (<10 mU H_2O_2/mg protein at 100 μg/ml nanaomycin A). And we found the IC_{50} value of the aerobic growth to be 6 μg/ml (data not shown), which are lower than IC_{50} values of NDH2 and MQO. Since IC_{50} of the succinate: quinone reductase activity would be over 30 μg/ml (data not shown), potential targets of nanaomycin A in *M. smegmatis* membranes are quinol: cytochrome *c* reductase, and/or cytochrome *bd*-type quinol oxidase. This possibility needs to be tested in future screening studies.

Structural Similarity Between NDH2 and MQO—K_m values for ubiquinone and inhibitor sensitivity indicate the structural similarity in two flavin adenine dinucleotide (FAD)-dependent membrane-bound dehydrogenases, NDH2 (NdhA) and MQO, which have similar molecular mass (48,980 and 54,783 Da, respectively) in *M. smegmatis* mc^2 155. MQOs can be divided into two subgroups (Fig. 6). Actinobacterial MQO including *M. smegmatis* belongs to a major bacterial MQO family (MQO1),

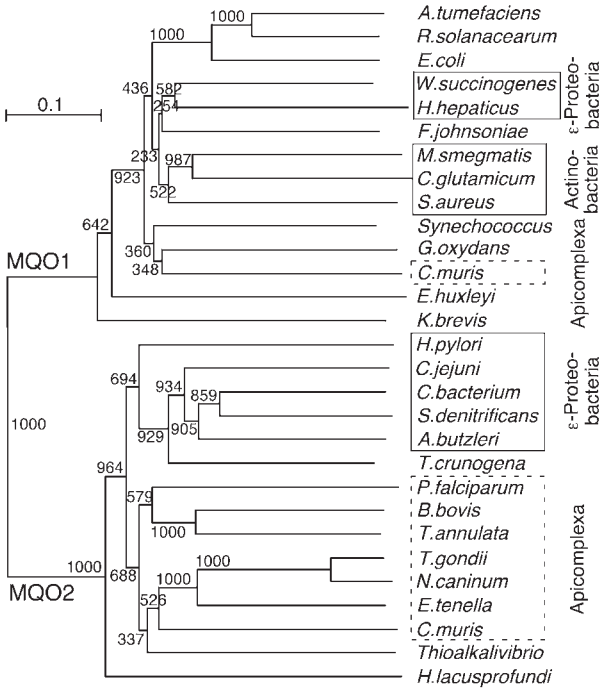

Fig. 6. **Phylogenetic analysis of MQO showing two subgroups.** MQO sequences (species, accession no.) used are apicomplexa [*Plasmodium falciparum*, XP_966154; *Toxoplasma gondii*, DQ457183; *Neospora caninum*, NC_LIV_111980*; *Eimeria tenella*, P_001238686*; *Babesia bovis*, XP_001611193; *Theileria annulata*, XP_955446; *Cryptosprodium muris*, FD413502** (MQO1) and XP_002139774 (MQO2)], Dinophyta (*Karenia brevis*, ABV49398**), Haptophyta (*Emiliania huxleyi*, GE148617**), α-proteobacteria (*G. oxydans*, YP_192462; *Agrobacterium tumefaciens*, Q8UH73), β-proetobacteria (*Ralstonia solanacearum*, YP_002254927), γ-proteobacteria (*E. coli*, P33940; *Thiomicrospira crunogena*, YP_392137; *Thioalkalivibrio* sp. HL-EbGR7, ZP_03277166), ε-proteobacteria (*Wolinella succinogenes*, NP_906991; *Helicobacter hepaticus*, NP_861251; *H. pylori*, NP_222801; *Campylobacterales bacterium*, EDZ61555; *Sulfurimonas denitrificans*, YP_394018; *Arcobacter butzleri*, YP_001489454; *Campylobacter jejuni*, ZP_03222725), cyanobacteria (*Synechococcus* sp. CC9311, YP_729866), Gram-positive bacteria (*Staphylococcus aureus*, P65423; *M*. smegmatis, YP_886950; *C. glutamicum*, O69282), Bacteroides (*Flavobacterium johnsoniae*, YP_001194875) and archaea (*Halorubrum lacusprofundi*, ZP_02017212). All sequences except for the sequences labelled with '*' are available from the GenBank. Sequences labelled with '*' are available from the GeneDB. Sequences labelled with '**' are ESTs and incomplete.

while most ε-proteobacterial MQO and apicomplexan mitochondrial MQO are major members of the second group (MQO2). As noted recently (*40*), phylogenetic analysis (Fig. 6) suggests that after the divergence of the chromoalveolata horizontal gene transfer of MQO2 from ε-proteobacteria to the apicomplexan parasites has occurred to likely facilitate the niche-specific adaptation to the host gastrointestine environments.

Here, we compared amino acid sequences of actinobacterial NDH2 and MQO1 (Fig. 7). Locations of conserved

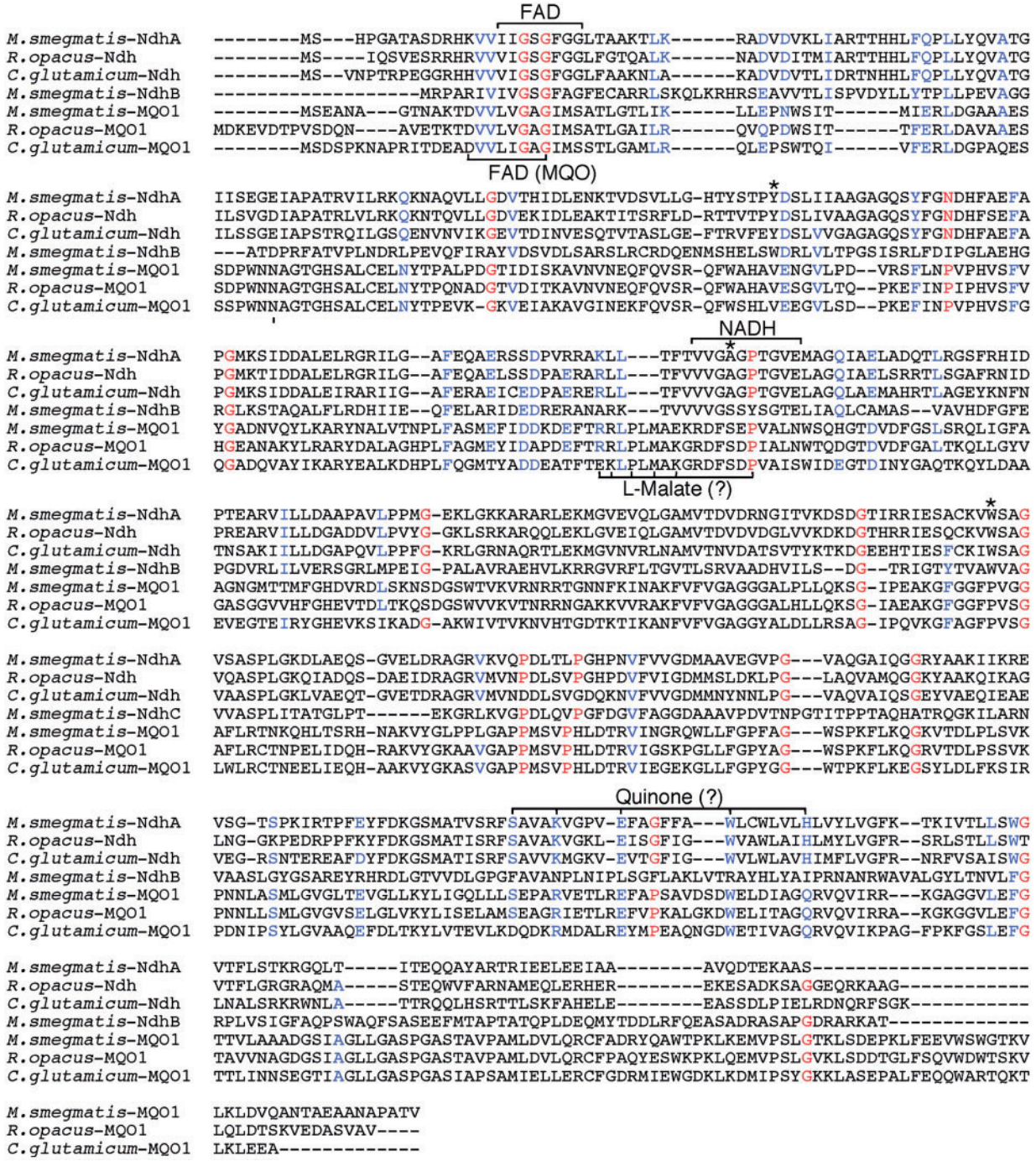

Fig. 7. **Sequence alignment of actionobacteria NDH2 and MQO1.** Sequences (GenBank accession no.) used are *M. smegmatis* mc^2 155 NdhA (YP_887924) and NdhB (YP_888708), *Rhodococcus opacus* B4 Ndh (YP_002784235), *Corynebacterium glutamicum* ATCC13032 (NP_600682), *M. smegmatis* MQO1 (YP_886950), *R. opacus* B4 MQO1 (YP_002783839) and *C. glutamicum* ATCC13032 MQO1 (NP_601207). Conserved helix breakers (Pro, Gly, Asn) are shown in red and other conserved amino acid residues are in blue. Proposed binding sites for FAD, NADH (*41–43*), L-malate and quinones (this study) are indicated by brackets. Locations of severe missense mutations (Y109H and A175S) (*10*) and Trp273 proposed for the quinone-binding (*45*) are indicated by asterisks.

helix breakers (Gly, Pro and Asn) and other amino acid residues indicate that they have likely evolved from a common ancestor. The amino acid sequence of *M. smegmatis* NdhB is divergent from orthologues and the binding motifs for both NADH and quinones are not conserved. Miesel *et al*. (*10*) have reported that single point mutations (Y109H and A175S) in *M. smegmatis* NdhA have reduced the NQR activity to 4% of the control. Thus, NdhB is less likely to contribute to the NQR activity of the *M. smegmatis* membrane. The N-terminal region of NDH2 contains two Rossman folds ('$GxGx_2G$' motifs of the ADP-binding βαβ fold). The first motif serves as the FAD-binding site and the second motif is used for the NADH binding (*41*, *42*). Molenaar *et al*. (*43*) proposed a Dx_4GxG overlapping with the first Rossman fold as the FAD-binding site in MQO. Near the NADH-binding site in NDH2, we found a possible malate-binding motif '$RRxPxMxKxRDx_3P$', which is similar to the malate-binding site ($R/Kx_2GMxRxDLx_3N$) of soluble malate dehydrogenase (*44*) in MQO. In the C-terminal region of NDH2, Vries *et al*. (*45*) proposed a possible role of Trp273 (Trp337 in *Saccharomyces cerevisiae* Ndi1) in the quinone binding. However, this Trp residue is not conserved in MQO. Inspection of the sequence alignment revealed a cluster of hydrophilic residues ($Sx_3K/Rx_{4\sim5}Ex_{6\sim9}Wx_6H/Q$), which could coordinate quinones in both NDH2 and MQO. We found recently the importance of K252 and E257 in quinol binding by subunit I of *E. coli bd*-type quinol oxidase (*46*). Abramson *et al*. (*47*) postulated that 'Rx_3D *plus* Hx_2Q' motif in subunit I of *E. coli bo*-type quinol oxidase coordinates a tightly bound ubiquinone. Putative binding sites for L-malate and quinones (Fig. 7) need to be tested in future site-directed mutagenesis studies.

CONCLUSION AND PERSPECIVES

Random screening of the natural antibiotics library with membrane-bound dehydrogenases (matrix screening) identified polymyxin B and nanaomycin A as inhibitors of *M. smegmatis* NDH2 and MQO. Regrettably, polymyxin B showed a poor bacteriocidal activity on *M. smegmatis*. Polymyxins are now re-evaluated as the last resort for infection with MDR Gram-negative bacteria (*27–29*). Synthetic modifications of polymyxins may improve the potential of peptide antibiotics (*48*). Parallel screening with *G. oxydans* NDH2 identified gramicidin S and scopafungin as potent inhibitors (*19*) and that with *P. aeruginosa*, membrane-bound dehydrogenases identified siccanin as a succinate dehydrogenase inhibitor (*49*). Variations in structures of a natural compounds library could allow us to identify species-specific inhibitors for the respiratory chain enzymes. We are hoping that continuing efforts on screening of the library with pathogen's enzymes could identify lead compounds for the development of new antibiotics targeting to the respiratory enzymes. Alternatively, inhibitors that we identified (*e.g*. gramicidin S, polymyxin B, scopafungin) are totally different from known respiratory complex inhibitors and they may have advantages in future functional studies (*e.g*. trapping the reaction intermediate).

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CONFLICT OF INTEREST

None declared.

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