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内視鏡関連感染対策

1 内視鏡室

1.1 施設で内視鏡検査を施行する部門を一カ所に集約する方が良い（内視鏡センター）[1]。(IIIB)

1.1.1 内視鏡の衛生管理と保守点検の責任者を置く[2-4]。(IVA)

1.1.2 内視鏡の運用（洗浄・消毒、個人用防護具の使い方、内視鏡の保管、検査室の清掃など）に関する手順を標準化する。(IIIA)

1.2 施設に適合した内視鏡の運用方法をマニュアル化する[2-4]。(IVA)

1.2.1 使用するたびに製造業者の取扱説明書に従って、加圧リーク・テストを実行する[1, 5-7]。(IA)

1.2.2 使用後の内視鏡は専用の搬送用トレイに入れ、周囲に汚染のないように洗浄室まで搬送する。(IIIA)

1.3 内視鏡室は、医療従事者や患者の安全を考慮して設計し、換気設備により有害な消毒薬の曝露を最小限化する[1, 5, 7-11]。(IA)

1.4 術者、介助者、洗浄する者が、個人用防護具をいつでも使用できるようにして、血液、化学物質、他の感染性物質に曝露されないようにする[12]。(IVA)

1.4.1 術者は検査中に清潔な手袋（未滅菌で良い）、ガウン、マスク、ゴーグル（またはフェイスシールド）を着用する[12]。(IIIA)

1.4.2 検査終了後、個人防護具を全て外し、手指衛生を行う。(IIIA)

1.4.3 個人用防護具をしたままカルテなどの記載は行わない。(IIIB)

1.4.4 介助者は必要に応じて個人用防護具を使用するが、患者ごとに個人用防護具を換える。(IIIB)

1.4.5 洗浄する者は手袋、ガウン、マスク、ゴーグル（またはフェイスシールド）を着用する。(IIIA)

1.4.6 洗浄終了後、個人用防護具を全て外し、手指衛生を行う。(IIIA)

1.4.7 洗浄中であっても個人用防護具をしたまま検査室から出ない。(IIIA)

1.5 内視鏡室に勤務する全ての職員は、感染管理上の推奨事項（例えば標準的な感染予防策）について訓練を受け、それを厳守する[13]。(IIIA)

1.5.1 内視鏡の衛生管理に関する院内研修会を開催する[3, 4]。(IVA)

1.5.2 独自に院内研修会を持ちにくい施設では、地域で連携して内視鏡の衛生管理に関する院外研修会に参加する（少なくとも年1回以上）[3, 4]。(IVA)

1.6 内視鏡が使用前のものか使用後のものか判別できるように、医療機関で取り決めをしておく。(IIIA)

1.6.1 内視鏡の保管、検査室、洗浄室の順で動線を設定し、使用前の内視鏡と使用後の内視鏡が交差しないようにする。(IIIB)

1.6.2 使用後の内視鏡は直ちに専用の搬送用トレイに入れ、洗浄室に搬送する。(IIIA)

1.7 患者名、診療録番号、手技名、術者、内視鏡シリアルナンバー、洗浄者、内視鏡洗浄消毒装置についての検査記録簿を作成し記録する[1-4, 8]。(IVA)

2 内視鏡の一次洗浄

2.1 内視鏡の部品（送気・送水と吸引バルブなど）を取扱説明書に従って取り外し、完全に酵素系洗剤に浸漬する[14, 15]。(IIIA)

2.2 酵素系洗剤は温度管理が重要であり、使用毎に廃棄する[5, 7]。(IIIA)

2.3 自動洗浄消毒器を使用する場合でも必ず一次洗浄を行う[1, 5-8, 16-18]。(IIIA)

2.4 内視鏡のチャンネル、部品、コネクタは、開口部の大きさに合ったブラシを用いて洗浄する[7, 17]。(IIIA)

2.4.1 洗浄用品は単回使用製品にするか、使用ごとに洗浄後、消毒する[7, 17]。(IIIA)

3 内視鏡の再処理（消毒／滅菌）

3.1 内視鏡は使用ごとに高水準消毒を行うか滅菌する[1, 5, 7, 8, 13, 16, 17, 19, 20]。(IIIA)

3.1.1 高水準消毒薬としてグルタラール製剤、フタラール製剤、過酢酸を使用する。(IIIA)

3.1.2 内視鏡に適合した消毒薬およびその濃度を取扱説明書に従って選択する[5, 7, 8, 17]。(IIIA)

3.2 再利用可能な生検鉗子は滅菌する[1, 5-8, 13, 16, 17, 21]。(IIIA)

3.3 浸漬法を行う場合、内視鏡や部品を高水準消毒薬に完全に浸漬する。高水準消毒薬が全てのチャンネルを満たしていることを確認する[1, 6-8, 16, 17]。(IB)

3.4 取扱説明書により内視鏡と自動洗浄消毒器の適合性について確認する[5, 8, 17]。(IB)

3.5 用手法で消毒する場合、高水準消毒の後に、滅菌水、濾過水、水道水のいずれかで内視鏡をすすぎ、チャンネルを洗い流して、消毒薬を除去する[1, 5, 6, 8, 16, 18, 22]。(IIIA)

3.5.1 内視鏡をすすいだ水は1回毎に排水する[1, 5, 6, 8, 16, 18, 22]。(IIIA)

3.5.2 チャンネルに消毒用のエタノールまたは70%イソプロパノールを通した後、送気して乾燥させる[1, 5, 6, 8, 16, 18, 22]。(IIIA)

3.6 高水準消毒薬については最小有効濃度を日常的に確認する[1, 5, 7, 8, 16, 20]。(IIIA)

3.6.1 使用開始前に消毒薬をチェックして結果を記録する。(IIIA)

3.6.2 化学的インジケータで有効濃度を下回っていた場合、その消毒薬は廃棄する[1, 5, 7, 8, 16, 20]。(IIIA)

3.6.3 自動洗浄消毒器に消毒薬をつぎ足す場合は、最初に入れた溶液を基準として期限を設定する（用手洗浄の場合も同様）。消毒薬を後からつぎ足しても使用期限は延長しない[7, 16, 23]。(IIIA)

3.7 送水ボトルとその連結チューブは、最低1日1回、高水準消毒または滅菌を行う。送水ボトルには滅菌水または水道水を入れる[1, 24, 25]。(IIIB)

4 内視鏡の保管

4.1 内視鏡は汚染しないように保管する[1, 7, 16, 17]。(IIA)

4.1.1 洗浄、消毒した内視鏡は、専用の保管庫で保管する（ケースに保管しない）。(IIIA)

4.1.2 保管庫のドアは閉めておく。(IIIB)

4.2 内視鏡は乾燥しやすいように垂直に立てて保管する（製造元の指示に従ってキャップ、弁、他の取り外し可能な器具は外しておく）[1, 5, 7, 16, 17, 26]。(IIA)

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病原体別感染拡大防止対策

1 多剤耐性菌

原則：多剤耐性菌による感染症を防止、低化させるためには、①耐性菌の早期検出、②感染源や感染経路の特定と予防策による伝播・感染拡大の防止、③抗菌薬の使用法に関する点検と見直し、の3点が重要であるが、②と③については、別章で詳しく論じられるため、本章では、文献的な報告があるものについて、その骨子を記述する。

1.1 バンコマイシン耐性腸球菌：VRE

1.1.1 VREによる感染症患者を減少させるまたは患者予後を改善するためには、保菌患者の増加を防ぐ方が良い[1-5]。(IIB)

1.1.2 ハイリスク患者を収容、治療する骨髄移植病棟などではVRE保菌者のスクリーニングと汚染・感染防止策（標準的な感染予防策、接触感染予防策）を実施する方が良い[6-9]。(IIB)

1.1.3 長期抗菌薬使用患者では定期的便培養を行う方が良い[10]。(IIB)

1.1.4 VREの保菌者の多いICUでは、汚染・感染防止策（標準的な感染予防策、接触感染予防策）をとる[11-13]。(IIA)

1.1.5 第3世代セファロスポリンやバンコマイシンの投与は、術後のVRE感染症のリスク因子になるため、予防投与は避ける方が良い[14-16]。(IIB)

1.2 メチシリン耐性黄色ブドウ球菌：MRSA

1.2.1 MRSA感染症を減少させるためには、MRSA感染症がリスクとなる患者を収容する病棟においてMRSAの保菌のスクリーニングを行う方が良い[17]。(IIB)

1.2.2 MRSA感染症を減少させるためには、保菌者、感染症患者の個室収容、汚染・感染防止策（標準的な感染予防策、接触感染予防策）を行う[18,19]。(IIA)

1.3 多剤耐性緑膿菌：MDRP

1.3.1 多剤耐性緑膿菌の感染症は、ICU患者の予後を悪化させるので、その発生や拡散を予防する[20-24]。(IIA)

1.3.2 抗菌薬の長期投与は多剤耐性緑膿菌の選択や定着を促進するため、行わない[25]。(IIA)

1.4 薬剤耐性菌全般

1.4.1 薬剤耐性菌による感染症を減少させるためには、看護師の配置を十分に行う方が良い[26,27]。(IIB)

2 結核菌

2.1 救急外来、剖検室などで、結核が疑われる患者に接する職員は、N95マスクを着用する。(IIIA)

2.2 外来の予診で結核が疑われる患者については、トリアージ（患者の予診に基づく疑似患者の選り分けと個室待機、優先診療）を行う。(IIIA)

2.3 活動性の結核症およびそれが疑われる患者を確認した場合は、医師は、直ちに、最寄りの保健所長に報告し、「結核症指定医療機関」に紹介し、転送する。(IVA)

2.4 活動性の結核患者に接した職員には健康診断を行い、感染した可能性のある職員に対しては予防投薬を行う。(IVA)

2.5 転送が困難な重症の排菌患者は陰圧空調の個室に収容し、患者に接する際は、手指衛生を励行しN95マスクを着用する。(IVA)

2.6 多剤耐性結核菌（MDR-TB、XDR-TB）と判定された場合は、直ちに、「結核症指定医療機関」の専門家に相談し対策を講じる。(IVA)

2.7 結核菌またはそれを含む臨床材料を扱う場合は、必要な防護具を付け安全キャビネットの中で操作する。(IVA)

3 バチルス属菌等非侵襲性環境細菌

3.1 バチルス属菌等の環境菌が血液培養で分離された場合は、輸液（ルート）、静脈注射薬などの汚染による可能性を第一に考慮し、調査と対策を行う[28,29]。(IIA)

3.2 バチルス属菌等の環境菌の、同時多発事例や急激な分離件数の増加を確認した場合は、環境汚染調査を実施する方が良い。(IIIB)

4 飛沫感染、空気感染で伝播するウイルス等

4.1 麻疹、水痘、インフルエンザ、SARS等、飛沫感染や空気感染で伝播拡散する病原体による院内感染には、伝播様式に応じた感染予防策を実施する。(IVA)

4.2 外来では、疑似患者については、トリアージを行う。(IIIA)

4.3 空気感染の感染源となりうる患者を入院させる場合は、陰圧空調の個室に収容する。(IVA)

4.4 SARSについては、疑似患者の段階で、「患者」とみなして必要な法的手続きと感染拡大防止策を講じる。(IVA)

5 ヒト-ヒト感染が極めて低いとされている病原体

5.1 レジオネラ、アスペルギルス、非結核性抗酸菌など、ヒトからヒトへ感染する頻度が極めて低いとされている病原体による感染症については、標準的な感染予防策を行う。(IIIA)

5.2 給水（湯）設備やシャワーの蛇口（連口）などでレジオネラが繁殖しないよう、定期的な消毒とともに、水温を20℃以下、遊離残留塩素濃度を0.2～0.4mg/L（ppm）程度に常時保つか、貯湯槽で60℃以上、吹き出し口で55℃以上の熱湯を給湯する[30,31]。(IVA)

5.3 レジオネラ感染症が施設内で発生した場合は、浴槽、リハビリ用プール、シャワーの蛇口（連口）などの湿潤箇所の培養検査と検出箇所の使用禁止、衛生管理を徹底する。(IIIA)

6 クロストリジウム・ディフィシル、ノロウイルスなど糞便、吐物を介して感染する病原体

6.1 クロストリジウム・ディフィシル：患者は個室収容かコホーティングを行い、汚染・伝播防止策（標準的な感染予防策、接触感染予防策）の徹底、排便介助、オムツ交換、糞便処理の際の手指衛生の徹底、通常洗剤により、手が触れる箇所の定期的拭き取りによる芽胞の物理的除去、高濃度汚染が疑われる場合は、次亜塩素酸ナトリウム液などを用いて消毒する。(IIA)

6.2 ガチフロキサシン、モキシフロキサシンの投与後に分離された株は、北米で流行している強毒型のBI/NAP1/027株か否か検査する方が良い。(IIB)

6.3 重篤な腸管感染症状を呈する患者では強毒型の BI/NAP1/027 株を想定し、培養検査を行う方が良い。(IIB)

6.4 ノロウイルスでは患者は個室収容かコホーティングし、汚染・感染防止策（標準的な感染予防策、接触感染予防策）を徹底する。(IIIA)

6.5 ノロウイルス感染症患者の下痢、嘔吐物の処理時には、ペーパータオルで物理的に拭き取り、その後、床の消毒には、塩素濃度が 0.02%（200ppm）の次亜塩素酸ナトリウム液を使用する。使用し汚染したペーパータオルなどは、0.1%（1,000ppm）の次亜塩素酸ナトリウム液を入れたビニール袋に封入し廃棄する。調理器具などの器物の消毒には、0.02%（200ppm）の濃度の溶液を用いる[32]。(IIA)

7 消毒薬に抵抗性を示す細菌

7.1 クロストリジウム・ディフィシル、バチルス属菌など芽胞を形成する菌種に対しては、一般の消毒処置が無効であるため、手が触れやすく、汚染されやすい箇所を通常洗剤を用いて定期的に物理的な拭き取りによる除染を行う。(IIA)

7.2 芽胞の汚染が想定され、消毒が必要と考えられる場合には、次亜塩素酸ナトリウムを含む消毒薬を用いた消毒を行う。(IIA)

7.3 クロルヘキシジンに抵抗性を示すバークホルデリア・セパシアなどのブドウ糖非発酵菌群によるアウトブレイクが発生した場合には、消毒薬の使用が使用説明書通りに行われているかの点検を行い、湿潤箇所の拭き取り検査、さらに消毒薬抵抗性株の出現を考慮して対策を行う方が良い。(IIIB)

8 食品を介して感染する可能性のある病原体

8.1 ノロウイルス、サルモネラ、腸管出血性大腸菌（O157 など）カンピロバクターなど、汚染された食品を介して感染する可能性のある病原体による感染症が同時多発した場合は、食中毒とともに院内感染の両面からの調査と対策を実施する。(IVA)

8.2 ノロウイルス：前述を参照。

8.3 サルモネラ、腸管出血性大腸菌（O157 など）など：標準的な感染予防策の励行、可能な場合は個室収容、下痢便、吐物の処理の際の汚染・感染拡大防止策を徹底する。(IVA)

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アウトブレイク対応策

1 対応組織

1.1 院内感染対策委員会

1.1.1 院内感染対策委員会はアウトブレイクが疑われる場合に報告を受ける体制を整備しておく[1, 2]。(IVA)

1.1.2 施設管理者または院内感染対策担当責任者は、アウトブレイクが疑われる場合、緊急に院内感染対策委員会を開催する[1, 2]。(IVA)

1.2 外部調査委員会

1.2.1 院内感染対策委員会で感染源・感染経路の特定が困難な場合は、保健所などの行政機関、関連学会などの協力のもと外部調査委員会を設置する。(IIIA)

1.2.2 外部調査委員会は、院内感染対策委員会の感染源・感染経路に関する調査結果と対策の妥当性を評価し、改善策を提言した後に公開する。(IIIA)

2 対応の基本手順

2.1 アウトブレイクの発生を疑った場合、以下の手順で対応を進める[3]。(IIIA)

① アウトブレイクの確認
② 範囲（病棟・期間）の確認とアウトブレイク症例の確定
③ 対応策の提示と実施
④ 感染源・感染経路に関する調査
⑤ アウトブレイク収束の確認

3 アウトブレイクの確認

3.1 アウトブレイクを疑う情報を収集できる報告システムを構築する[1, 2]。(IVA)

3.2 院内感染対策委員会は、アウトブレイクを疑った場合、過去の発生状況及び原因病原体の分離や患者の迅速診断、血清診断結果に基づきアウトブレイクの発生を確認する。(IIIA)

4 アウトブレイクの範囲とアウトブレイク症例の確定

4.1 院内感染対策委員会が未把握の感染症 / 無症状病原体保有患者の有無を以下の方法で確認し、対応および調査の対象とする病棟及び期間を定める。(IIIA)

4.1.1 薬剤耐性菌による事例の場合には保菌検査を行う。(IIIA)

4.1.2 薬剤耐性菌の保菌検査は症例と同一の看護単位の全入院患者を対象とした方が良い。(IIIB)

4.1.3 アウトブレイク症例を明確にするため、アウトブレイク症例の定義を作成する（IIIA）

4.1.4 アウトブレイク症例の定義には、①アウトブレイクの対象となる感染症・無症状病原体保有患者の定義、②アウトブレイクの発生期間、③アウトブレイクの対象となる病棟、の3項目を含める方が良い。(IIIB)

5 対応

5.1 初期対応

5.1.1 アウトブレイクの原因病原体の伝播経路に応じて症例の隔離を実施する。(IIIA)

5.1.1.1 処理病原体に応じた設備が整備された病室で個室またはコホート管理を実施する。(IIIA)

5.1.2 対象となる病棟の全ての入院患者の湿性生体物質に触れる処置を行う場合には、清潔な手袋(未滅菌で良い)・マスク・ガウン等の使用と手指の衛生管理を徹底・強化する。(IIIA)

5.1.3 対象となる病棟における手指衛生管理の実施を徹底・強化する。(IIIA)

5.1.4 症例の隔離のため、職員に対する感染対策に関する情報提供を行った方が良い。(IIIB)

5.1.5 医療器材の滅菌と消毒・使用薬剤の衛生管理、および処置時の衛生管理を再度見直す。(IIIA)

5.1.6 予防薬やワクチンがある場合は、その適応を考慮する方が良い[4, 5]。(IIIB)

5.2 初期対応後の対応

5.2.1 初期対応の有効性を評価するため、新規のアウトブレイク症例の有無を継続的に監視する。(IIIA)

5.2.2 新規のアウトブレイク症例が発生した場合、初期対応を講じた後も病原体の伝播が阻止されていない可能性があるので以下の対応を行う。

5.2.2.1 感染源、感染経路に関する調査を実施し随時対策を追加する。(IIIA)

5.2.2.2 感染源、感染経路の特定が困難な場合は以下のような外部専門家の支援を要請する。(IIIA)

①保健所や地方衛生研究所

②各都道府県を通じて要請する国立感染症研究所実地疫学専門家養成プログラム(FETP)

③院内感染対策地域支援ネットワーク事業

④大学等の医育機関

⑤感染症関連学会等

5.2.2.3 当該病棟への新規入院の中止等、診療を制限する方が良い[6]。(IIIB)

5.3 事例後対応

5.3.1 継続的な監視を行っても新規の症例発症が一定期間＊認められなかった場合には、アウトブレイクの収束と判断して良い。(＊一定期間:一般的には潜伏期間の2～3倍の期間)(IIIC)

5.3.2 アウトブレイクの収束が確認された後、感染源、感染経路に関しての調査結果を参考に、一時的に強化していた種々の対策を継続可能な対策に切り替えていく。(IIIA)

6 調査

6.1 事例の早期収束および再発防止のため、アウトブレイク症例に共通する感染源・感染経路に関する調査を行う。(IIIA)

6.2 病原体・患者検体の確保および検査

6.2.1 分離された病原体や採取された血清などの検体は保管する[7, 8]。(IIIA)

6.2.2 環境消毒の前に環境調査を行う。(IIIA)

7 情報の公開

7.1 事例の概要、感染源・感染経路に関する調査内容、改善策をまとめ、患者の了解を得た後に公開する。(IIIA)

8 患者・家族・医療従事者への情報提供

8.1 入院患者およびその家族に対しては、初期対応時に診療担当者と院内感染対策の担当者等から十分な情報を提供し対策に必要な協力を要請する。(IIIA)

8.2 全ての職員に対して、院内感染対策委員会よりアウトブレイク事例ならびにその対策に関する情報を提供する。(IIIA)

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Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表(１)

著者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Wachino J., Shibayama K., Kurokawa H., Kimura K., Yamane K., Suzuki S., Shibata N., Ike Y., and Arakawa Y.	Novel Plasmid-Mediated 16S rRNA m1A1408 Methyltransferase, NpmA, Found in a Clinically Isolated *Escherichia coli* Strain Resistant to Structurally Diverse Aminoglycosides	Antimicrob Agents Chemother.	51(12)	4401-4409	2007
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Kimura K, Suzuki S, Wachino J, Kurokawa H, Yamane K, Shibata N, Nagano N, Kato H, Shibayama K, Arakawa Y	Emergence of Group B Streptococci with Reduced Penicillin-Susceptibility.	Antimicrob Agents Chemother	in revision		
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Yamane K, Wachino J, Suzuki S, Shibata N, Kato H, Shibayama K, Kimura K, Kai K, Ishikawa S, Ozawa Y, Konda T, Arakawa Y	16S rRNA methylase-producing, gram-negative pathogens, Japan	Emerg Infect Dis.	13(4)	642-6	2007
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Makino M , Maeda Y., Fukutomi Y., Mukai T	Contribution of GM-CSF on the enhancement of the T cell-stimulating activity of macrophages.	Microbes and Infect.	9	70-77	2007

研究成果の刊行に関する一覧表(2)

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Novel Plasmid-Mediated 16S rRNA m^{1}A1408 Methyltransferase, NpmA, Found in a Clinically Isolated *Escherichia coli* Strain Resistant to Structurally Diverse Aminoglycosides$^{\nabla}$

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We have isolated a multiple-aminoglycoside-resistant *Escherichia coli* strain, strain ARS3, and have been the first to identify a novel plasmid-mediated 16S rRNA methyltransferase, NpmA. This new enzyme shared a relatively low level of identity (30%) to the chromosomally encoded 16S rRNA methyltransferase (KamA) of *Streptomyces tenjimariensis*, an actinomycete aminoglycoside producer. The introduction of a recombinant plasmid carrying *npmA* could confer on *E. coli* consistent resistance to both 4,6-disubstituted 2-deoxystreptamines, such as amikacin and gentamicin, and 4,5-disubstituted 2-deoxystreptamines, including neomycin and ribostamycin. The histidine-tagged NpmA elucidated methyltransferase activity against 30S ribosomal subunits but not against 50S subunits and the naked 16S rRNA molecule in vitro. We further confirmed that NpmA is an adenine N-1 methyltransferase specific for the A1408 position at the A site of 16S rRNA. Drug footprinting data indicated that binding of aminoglycosides to the target site was apparently interrupted by methylation at the A1408 position. These observations demonstrate that NpmA is a novel plasmid-mediated 16S rRNA methyltransferase that provides a panaminoglycoside-resistant nature through interference with the binding of aminoglycosides toward the A site of 16S rRNA through N-1 methylation at position A1408.

Aminoglycosides such as kanamycin, gentamicin, and neomycin bind to the A site of the 16S rRNA of the bacterial 30S ribosomal subunit and subsequently block growth through interference with protein synthesis (25). These agents have been used for the treatment of a broad range of life-threatening infections due to both gram-positive and gram-negative bacteria in human and veterinary medicine (18, 37). However, bacteria have acquired various aminoglycoside resistance mechanisms, such as through the production of aminoglycoside-modifying enzymes (acetyltransferase, nucleotidyltransferase, and phosphotransferase), the reduction of antibiotic penetration on the outer membrane protein, the acquisition of reduced affinity by changing key nucleotides within the 16S rRNA, and augmented excretion by an efflux pump system (5, 25, 36, 42).

In 2003, a plasmid-mediated 16S rRNA methyltransferase, which confers a high level of resistance to various clinically important aminoglycosides, was reported to be involved as part of a novel aminoglycoside resistance mechanism in pathogenic gram-negative rods (16, 53). At present, five types of plasmid-mediated 16S rRNA methyltransferase genes, *rmtA*, *rmtB*, *rmtC*, *rmtD*, and *armA*, have been found worldwide in members of the family *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and *Acinetobacter* spp. (4, 7, 10, 11, 17, 20, 34, 48, 50–52). Also, these genes are mediated by bacterium-specific recombination systems, such as transposons, and are easily translocated to other DNA target sites (17, 19, 47, 49).

The 16S rRNA methyltransferases conferring aminoglycoside resistance are supposed to have evolved as a self-defense mechanism in aminoglycoside-producing actinomycetes, including *Streptomyces* spp. and *Micromonospora* spp (9). The methylation of 16S rRNA plays a crucial role in prevention of the adverse effects of intrinsic aminoglycosides that would block their own 16S rRNA. The 16S rRNA methyltransferase conferring aminoglycoside resistance consists of two different groups, one methylates the N-7 position of G1405 and confers panresistance to aminoglycosides belonging to both the kanamycin and the gentamicin groups (3, 44), and the other methylates the N-1 position of A1408 and provides resistance to kanamycin and apramycin (3, 22, 43). Recently, it was reported that the plasmid-mediated 16S rRNA methyltranferase ArmA methylates the N-7 position of G1405 within 16S rRNA (27). On the other hand, no plasmid-mediated 16S rRNA methyltransferase which modifies the N-1 position of A1408 has so far been found in any pathogenic bacteria isolated from clinical settings and natural environments. Therefore, we screened for a new plasmid-mediated methyltransferase that methylates A1408 among bacterial species belonging to the family *Enterobacteriaceae*, *P. aeruginosa*, and *Acinetobacter* spp. isolated in Japanese clinical settings. Apramycin resistance seemed to be a good indicator for the detection of an A1408 16S rRNA methyltransferase producer, since a previous study reported that the introduction of a recombinant plasmid encoding a gene for the A1408 16S rRNA methyltransferase derived from a *Streptomyces* sp. was also able to confer a high level of

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype and phenotype	Source or reference
E. coli strain		
ARS3	Clinical isolate	This study
CSH-2	*metB* F⁻, nalidixic acid resistant, rifampin resistant	Laboratory strain
JM109	*endA1 recA1 gyrA96, thi-1 hsdR17*(r_K^- m_K^+) *relA1 supE44* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}*ZΔM15*]	Takara
BL21(DE3)pLysS	F⁻ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* λ(DE3)pLysS (chloramphenicol resistant)	Novagen
Plasmid		
pARS3	115-kb conjugative plasmid carrying *npmA* of *E. coli* ARS3	This study
pMCL-H	Plasmid carrying 3,946 bp fragment containing *npmA* on pMCL210	This study
pMCL-BE	Plasmid carrying 980-bp fragment containing *npmA* on pMCL210	This study
pMCL-BH	Plasmid carrying *npmA* tagged with five histidine codons at its 3′ end and its promoter region on pMCL210	This study
pBCE	Plasmid carrying an EcoRI-digested fragment containing *npmA* on pBCSK+	This study
pBCSII	Plasmid carrying a SacII-digested fragment containing *npmA* on pBCSK+	This study
pCold-NpmA	Plasmid carrying *npmA* ligated to pCold-IV	This study
pMCL210	Cloning vector, chloramphenicol resistant	30
pBCSK+	Cloning vector, chloramphenicol resistant	Stratagene
pCold-IV	Protein expression vector, ampicillin resistant	Takara

resistance to apramycin (43). The use of this screening protocol on the basis of apramycin resistance allowed us to identify a panaminoglycoside-resistant *Escherichia coli* strain, strain ARS3, that produces a novel plasmid-mediated methyltransferase, newly assigned NpmA, that methylates A1408 at the A site of 16S rRNA. The aim of this study was to characterize the molecular mechanism underlying the panaminoglycoside resistance conferred by NpmA.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strain ARS3 was isolated in 2003 from the urine of an inpatient in a general hospital in Japan. The bacterial strains were grown in LB broth at 37°C with shaking, unless otherwise indicated. MICs were determined by the agar dilution method with Mueller-Hinton agar plates, according to the protocol recommended by the Clinical and Laboratory Standards Institute (8).

Conjugation. A conjugation experiment was performed as described elsewhere (48). *E. coli* strain CSH-2 was used as the recipient. Conjugants were selected on LB agar plates containing apramycin at 150 μg/ml and rifampin at 100 μg/ml.

Cloning of *npmA*. The transferable plasmid pARS3 was extracted from the *E. coli* conjugant and digested with restriction enzymes. The resultant fragments were ligated to cloning vectors and electroporated into *E. coli* strain JM109. The transformants were selected on LB agar plates supplemented with apramycin at 150 μg/ml and chloramphenicol at 30 μg/ml. The *npmA* gene was amplified with primer P1 (5′-CGG GAT CCA AGC ACT TTC ATA CTG ACG-3′) and primer P2 (5′-CGG AAT TCC AAT TTT GTT CTT ATT AGC-3′) (the underscored sequences indicate BamHI and EcoRI restriction sites, respectively) and cloned into the vector pMCL210.

N-terminal determination of NpmA. The DNA fragment carrying *npmA* and its promoter region was amplified by PCR with primers P1 and P3 (the sequence of primer P3 is 5′-CCC AAG CTT TTA atg atg atg atg atg ATG TTT TGA AAC ATG GCC-3′ [where the underscores indicate the Hind III restriction site and the sequence with lowercase letters represents the nucleotide sequence of C-terminal histidine tag]). Primer P3 was designed so that five histidine codons could be added to the 3′ end of *npmA*. The resultant fragments were ligated to pMCL210 and introduced into *E. coli* JM109. The cells were cultured in 1 liter of LB broth containing chloramphenicol at 30 μg/ml, disrupted with a French press, and centrifuged at 100,000 × *g* for 1 h. The supernatant containing the recombinant protein was loaded onto a HisTrap HP column (Amersham Biosciences) and purified according to the manufacturer's instructions. The N-terminal sequence of the purified protein was obtained by Edman degradation in a model Shimadzu PPSQ-23 automated protein sequencer.

Overexpression and purification of histidine-tagged NpmA. The *npmA* gene was amplified with primer P4 (5′-GGA ATT CCA TAT GTT AAT ACT CAA AGG AA-3′), which introduced an NdeI restriction site at the 5′ end, and primer P3, which introduced a HindIII restriction site and five histidine codons at the 3′ end. The amplified fragments were cloned into the pCold-IV vector (Takara) and introduced into *E. coli* BL21(DE3)pLysS. The purification of recombinant protein was performed as described above, with some modifications. After the step of nickel-nitrilotriacetic acid chromatography, the eluted protein was dialyzed against 50 mM sodium phosphate buffer (pH 6.4). Furthermore, the protein was applied to a cation-exchange HiTrap S HP column (Amersham Biosciences). Finally, the eluted protein was concentrated and the buffer was exchanged with 50 mM sodium phosphate buffer (pH 7.4).

Methylation assay. Both the 30S and the 50S subunits of *E. coli* JM109 were prepared as described previously (27). After ultracentrifugation with 10 to 30% sucrose density gradients, the 30S and 50S subunit fractions were collected. The purity of each subunit was checked by denatured agarose gel electrophoresis of the rRNA derived from the material. The methylation assay was carried out at 35°C, as follows. Thirty picomoles of substrate, 30 pmol of His_5-NpmA, and 7.5 μCi of *S*-adenosyl-L-[*methyl*-^{3}H]methionine (76 Ci/mmol, 1 mCi/ml) were adjusted to 300 μl with methylation buffer (50 mM HEPES-KOH, pH 7.5; 10 mM $MgCl_2$; 100 mM NH_4Cl; 5 mM 2-mercaptoethanol). Aliquots were taken at 0, 5, 15, 30, and 45 min and purified with an RNeasy mini kit (Qiagen), according to the instructions provided by the manufacturer. The samples were counted with a scintillation counter.

RNase protection assay. One picomole of [^{3}H]methyl-labeled 16S rRNA was hybridized with 100 pmol of a deoxyoligonucleotide (positions 1421 to 1392 [5′-CAC TCC CAT GGT GTG ACG GGC GGT GTG TAC-3′] and positions 1507 to 1478 [5′-TAC CTT GTT ACG ACT TCA CCC CAG TCA TGA-3′]) in 50 μl of hybridization buffer (40 mM morpholineethanesulfonic acid, pH 6.4; 400 mM NaCl; 9 mM EDTA; 80% [vol/vol] formamide) The sample was incubated at 90°C for 10 min, cooled at room temperature for 15 min, and diluted with 450 μl of RNase buffer (10 mM Tris-HCl, pH 7.5; 300 mM NaCl; 5 mM EDTA) containing RNase T_1 (Roche). The digestion was performed at 37°C for 1 h. The reaction was stopped by adding 4.5 ml of 10% ice-cold trichloroacetic acid, and the reaction mixture was placed on ice for 10 min. The samples were passed through cellulose nitrate filters. The filters were dissolved in scintillation fluid, and the radioactivity was measured.

Primer extension. One microgram of 16S rRNA extracted from the 30S subunits methylated in vitro was hybridized with 50 pmol of a primer (5′-biotin-CCA ACC GCA GGT TCC CCT ACG G-3′) complementary to nucleotides 1530 to 1509 at 65°C for 10 min. The elongation was performed with Transcriptor reverse transcriptase (Roche) at 43°C for 1 h. The cDNA transcripts were analyzed on an 8% polyacrylamide gel containing 8 M urea.

HPLC assay of methylated adenine residue. 16S rRNA was extracted from the 30S subunits of *E. coli*. Sixty micrograms of extracted 16S rRNA was digested with nuclease P1 (3 U; Wako) and alkaline phosphatase (0.08 U; Takara) in 120 μl of a reaction mixture containing 25 mM HEPES-KOH (pH 7.5) at 37°C for 6 h. The resulting mixture was analyzed by high-performance liquid chromatog-

TABLE 2. Antimicrobial susceptibilities of parental strain, transconjugant, and transformant

Aminoglycoside	MIC (μg/ml)				
	E. coli ARS3(pARS3)	*E. coli* CSH-2(pARS3)	*E. coli* CSH-2	*E. coli* JM109(pMCL-BE)	*E. coli* JM109(pMCL210)
4,6-Disubstituted 2-deoxystreptamines					
Kanamycin group					
Arbekacin	64	4	0.13	4	0.25
Amikacin	256	16	0.13	32	0.5
Dibekacin	>256	128	0.13	128	0.25
Kanamycin	>256	>256	0.25	>256	0.5
Tobramycin	>256	128	≤0.06	128	0.13
Gentamicin group					
Gentamicin	>256	128	≤0.06	16	0.13
Isepamicin	>256	64	0.13	128	0.13
Netilmicin	>256	>256	≤0.06	>256	0.13
Sisomicin	>256	>256	≤0.06	256	0.13
4,5-Disubstituted 2-deoxystreptamines					
Lividomycin A	256	16	0.5	32	2
Neomycin	>256	64	0.13	64	0.25
Paromomycin	64	4	0.5	4	1
Ribostamycin	>256	>256	0.25	>256	0.5
Other aminoglycosides					
Apramycin	>256	>256	1	>256	2
Hygromycin B	128	16	16	32	32
Streptomycin	128	32	1	1	1
Spectinomycin	>256	>256	16	16	16

raphy (HPLC) with an HRC-ODS column (4.6 mm [inner diameter] by 250 mm; Shimadzu). The solvent system consisted of 5 mM ammonium acetate (pH 5.3) (solvent A) and 30% acetonitrile (solvent B) and was used as follows: 0% to 50% solvent B from 0 to 100 min, 50% to 99% solvent B from 100 to 110 min, and 99% solvent B from 110 to 130 min, with an effluent rate of 600 μl/min at 30°C.

Aminoglycoside binding to 30S subunit. Sixty picomoles of the wild-type or the modified 30S subunits was incubated in 100 μl of dimethylsulfate (DMS) buffer (80 mM sodium cacodylate, pH 7.2; 100 mM NH_4Cl; 20 mM $MgCl_2$; 1 mM dithiothreitol; 0.5 mM EDTA) at 42°C for 10 min. Addition of aminoglycosides (final concentration range, 1 μM to 1,000 μM) was followed by incubation at 37°C for 30 min and then on ice for 10 min. DMS (2 μl, 1:6 in ethanol) was added, and the mixture was incubated at 37°C for 10 min. The reaction was quenched by adding 25 μl of stop buffer (1.5 M sodium acetate, 1 M 2-mercaptoethanol). After ethanol precipitation, modified rRNA was obtained by extraction with phenol three times and chloroform twice. Reduction with sodium borohydride and aniline-induced strand scission were performed as described previously (27). A primer extension analysis was carried out as described above.

Nucleotide sequence accession number. The open reading frame of *npmA* was deposited in the EMBL and GenBank databases through the DDBJ database and has been assigned accession number AB261016.

RESULTS

Characteristics of *E. coli* strain ARS3. The MICs of various aminoglycosides for parent *E. coli* strain ARS3 are shown in Table 2. This strain demonstrated resistance to structurally diverse aminoglycosides. The panaminoglycoside-resistant phenotype of strain ARS3 was successfully transferred to the *E. coli* CSH-2 recipient strain at a frequency of 2×10^{-8} per donor by conjugation. The transconjugant acquired a transferable plasmid (pARS3), which was estimated to be about 115 kb in size by summation of the sizes of the EcoRI digestion products, and demonstrated resistance to various aminoglycosides (Table 2).

Genetic determinant of aminoglycoside resistance on transferable plasmid pARS3. A cloning experiment was performed to confirm the genetic aminoglycoside resistance determinant, which is mediated by pARS3. As a result, one recombinant plasmid (plasmid pMCL-H) was obtained by selection with apramycin and chloramphenicol, and both strands of the 3,946-bp HindIII insert were entirely sequenced. The schematic organization of probable genes found in the cloned fragment is shown in Fig. 1. To identify the gene responsible for apramycin resistance, Tn*5* (Tet^r) insertion mutants of clone pMCL-H were generated. A total of 12 insertion mutants were obtained (Fig. 1), and 3 of them that carried a Tn*5* insertion in *orf6* lost apramycin resistance. Recombinant plasmid pMCL-BE, which contained only *orf6* and its putative promoter region, showed apramycin resistance, as was seen in clone pMCL-H.

The deduced amino acid sequences of ORF6 exhibited low-level identities (<31%) to the chromosomally encoded 16S rRNA methyltransferases KamA, KamB, KamB2, KamB3, KamC, and Amr of actinomycetes that produce aminoglycosides. Several studies already revealed that some of these 16S rRNA methyltransferases of actinomycetes methylate the N-1 position of nucleotide A1408 in 16S rRNA and confer intrinsic aminoglycoside resistance to bacteria (3, 22, 43). Therefore, it is probable that the product of *orf6*, NpmA, has 16S rRNA methyltransferase activity and confers panresistance to aminoglycosides in a manner similar to that seen in aminoglycoside-producing actinomycetes. NpmA has a conserved residue (D) and the consensus GXGXG motif, which is considered the hallmark *S*-adenosylmethionine (SAM)-binding site of Ross-

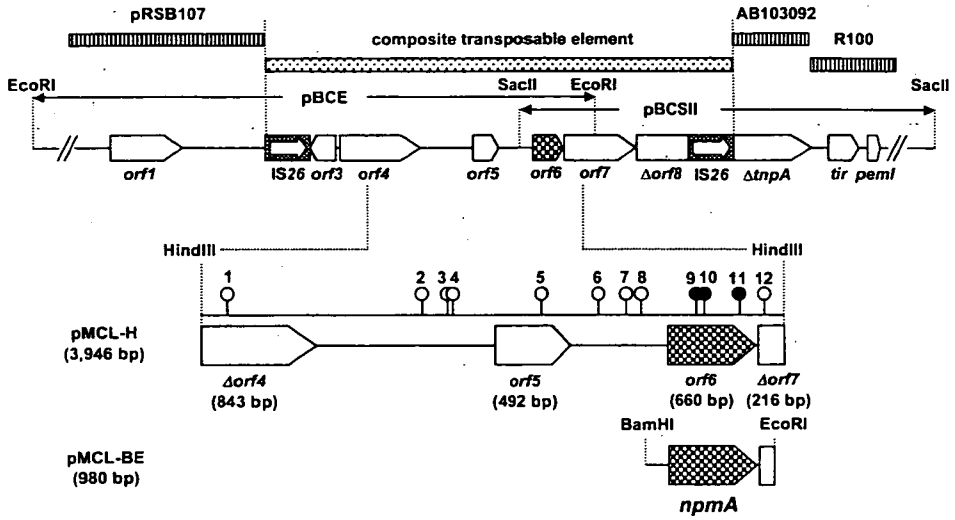

FIG. 1. Schematic presentation of open reading frames (ORFs) in the cloned fragment conferring aminoglycoside resistance. Open reading frames are shown as arrows indicating the transcription orientation. The positions where Tn*5* was inserted are indicated by open and closed circles. Mutants with Tn*5* insertions shown by open circles demonstrated aminoglycoside resistance, while those indicated by closed circles did not.

man fold SAM-dependent methyltransferases (Fig. 2) (26). SAM is often used as the source of the methyl group in methyltransferase reactions in various organisms (26).

Genetic environments of *npmA*. The structures of the flanking regions of *npmA* were determined (Fig. 1). The genes for *orf7* (which encodes a probable ABC transporter substrate binding protein) and *orf8* (which encodes a truncated mobilization protein) were located at the 3′-end region of *npmA*. Three open reading frames, *orf3* (which encodes a hypothetical protein), *orf4* (which encodes a possible replication protein), and *orf5* (which encodes a hypothetical protein), existed at the 5′-end region of *npmA*. The 9.1-kb region containing *orf3* to *orf8* was flanked by two IS*26* elements in direct orientation and composed a transposable element (12). The sequences around the 9.1-kb transposable element have significant sequence similarities to the sequences of a part of various multidrug resistance plasmids deposited in the EMBL/GenBank/DDBJ databases.

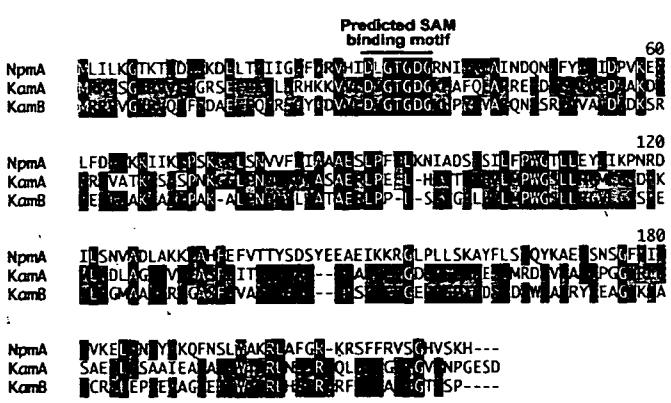

FIG. 2. Alignment of deduced amino acid sequences of NpmA with those of KamA and KamB. Chromosomal 16S rRNA methyltransferases (KamA and KamB) were found in aminoglycoside-producing actinomycetes (24, 32). Identical amino acids in all proteins are highlighted with a dark background. Physicochemically similar amino acids are highlighted with a gray background.

Antibiotic susceptibilities. The MICs of the aminoglycosides for the NpmA-producing *E. coli* transformant are shown in Table 2. The introduction of *npmA*-carrying plasmid pMCL-BE conferred resistance to both 4,6-disubstituted 2-deoxystreptamines, consisting of the kanamycin and gentamicin groups, and 4,5-disubstituted 2-deoxystreptamines, including neomycin and ribostamycin. In addition, NpmA augmented the MIC of apramycin, whose structure is far different from those of the 4,6- and 4,5-disubstituted 2-deoxystreptamines. On the other hand, NpmA did not confer resistance to the non-A-site binders streptomycin and spectinomycin. On the whole, NpmA could confer resistance to various aminoglycosides which bind to the A site of the decoding region in 16S rRNA.

N-terminal sequence of NpmA. As shown in Fig. 2, the exact locations of the N termini of A1408 methyltransferases are still controversial. For example, the N-terminal position of the Kam family of proteins, including KamB and KamC, was previously reported to be position M61, shown in Fig. 2 (22). This fact, however, indicates the lack of a SAM-binding motif, which plays a crucial role in methyltransferase activity among the mature Kam family of enzymes. Most recently, Koscinski et al. reanalyzed the amended amino acid sequences of a Kam family protein and revealed that the SAM-binding motif is perfectly conserved in the missing N-terminal sequences of the Kam family of proteins (24). In this study, in order to determine the exact position of the N terminus in NpmA experimentally, the recombinant NpmA protein was purified from *E. coli* cells harboring pMCL-BH and was subjected to Edman protein sequencing. The N terminus of NpmA was exactly determined to be MLILK (Fig. 2), although TTG is uncommon as a bacterial initiation codon.

Methylation of 30S subunits by NpmA. *E. coli* BL21 (DE3)pLysS and the pCold-IV expression vector were used for

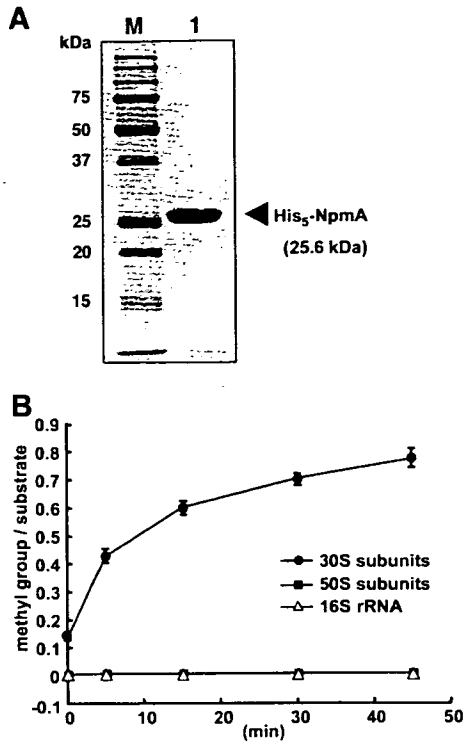

FIG. 3. Purification of NpmA and methylation assays. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified His_5-NpmA protein. Lanes: M, protein size marker; 1, purified His_5-NpmA. (B) Methyl acceptor activities of 30S subunits, 50S subunits, and naked 16S rRNA determined with [^{3}H]SAM and His_5-NpmA. The square symbols for the 50S subunits are hidden behind the triangle symbols for 16S rRNA.

the overexpression and purification of NpmA. *E. coli* BL21 (DE3)pLysS carrying pCold-IV is susceptible to apramycin (MIC, 3.9 μg/ml), while *E. coli* BL21(DE3)pLysS carrying pCold-NpmA exhibited a very high level of resistance to apramycin (MIC, >1,000 μg/ml) in the microdilution susceptibility test. This result indicated that the histidine-tagged NpmA (His_5-NpmA) still has methylation activity and is responsible for apramycin resistance in *E. coli* BL21(DE3)pLysS. An optimized culture condition yielded 8 mg of purified protein per 1 liter of bacterial culture, and the purified enzyme gave a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with Coomassie brilliant blue staining (Fig. 3A).

The methylation assay was performed with three different substrates, 50S subunits, 30S subunits, and 16S rRNA dissociated from the 30S subunits, to explore the activity of NpmA and its substrate specificity. NpmA was able to incorporate about 0.8 pmol of methyl groups into 1 pmol of 30S subunits after 45 min of incubation, whereas no significant incorporation of methyl groups into each 50S subunit or the dissociated 16S rRNA molecule was detected under the same experimental conditions (Fig. 3B). These results demonstrated that NpmA has optimal methyltransferase activity toward the properly assembled 30S subunit.

RNase protection assay. As described above, the in silico analysis indicated that NpmA exhibits amino acid sequence similarity to various chromosomally encoded A1408 16S rRNA

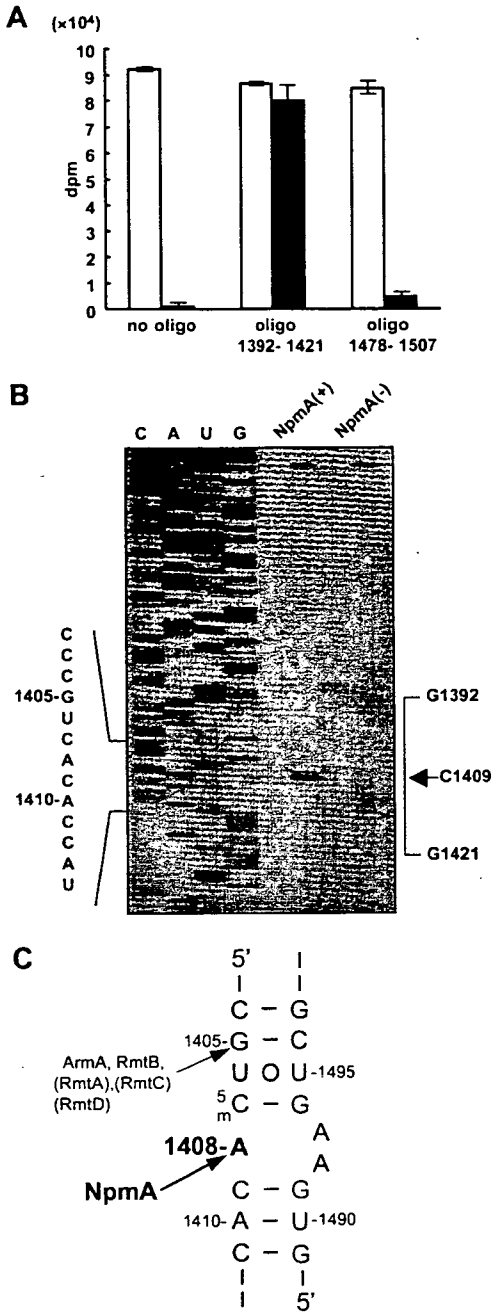

FIG. 4. Nuclease protection assay and primer extension analysis. (A) Nuclease protection assay with [^{3}H]-methyl-labeled 16S rRNA and DNA oligonucleotides (oligo) complementary to the regions from positions 1392 to 1421 or positions 1478 to 1507. The values are the averages of three measurements. Error bars indicate standard deviations. Open bars, undigested; solid bars, digested with RNase T_1. (B) Primer extension analysis of methylated 16S rRNA [NpmA(+)] and wild-type 16S rRNA [NpmA(−)]. Dideoxy sequencing lanes (C, A, U, and G) were generated with the amplified PCR products of *E. coli* 16S rRNA gene as the template. Primer extension termination at position C1409 is indicated by an arrow. (C) Methylation sites in the decoding region in 16S rRNA from *E. coli*. The exact methylation site by ArmA and RmtB was confirmed at G1405, but that by RmtA, RmtC, and RmtD has not yet been confirmed.

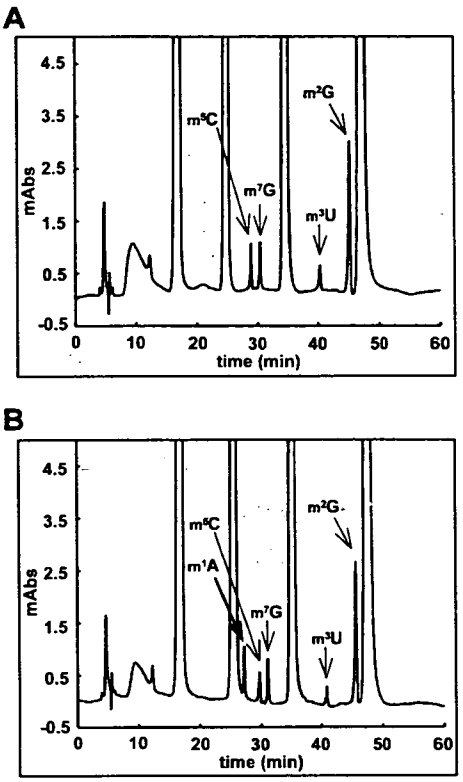

FIG. 5. HPLC analysis of methylated adenine residue. Purified 16S rRNA was completely digested with nuclease P1 and alkaline phosphatase and subjected to HPLC. (A) Wild-type 16S rRNA; (B) 16S rRNA methylated by NpmA. The m^1A peak indicates the formation of a methylated adenosine residue at 1408. mAb, milliabsorbance units.

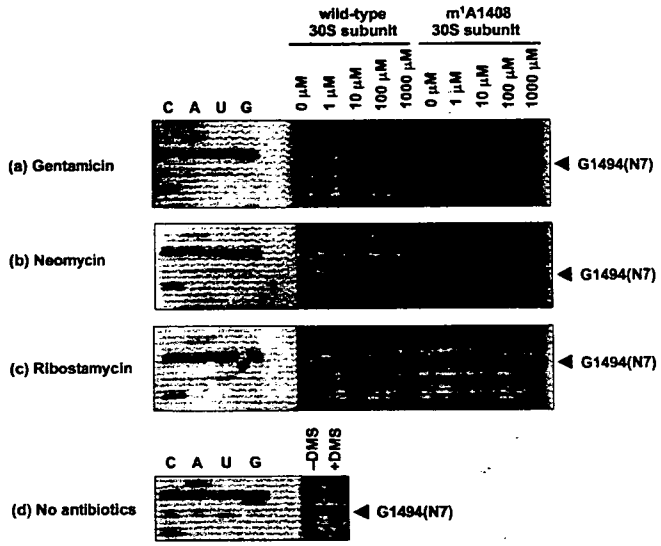

FIG. 6. Footprinting for confirmation of aminoglycoside binding to 30S subunits. The gentamicin, neomycin, and ribostamycin footprints are indicated at the position of G1494 (N-7) in the 16S rRNA. Dideoxy sequencing lanes (lanes C, A, U, and G) were generated with amplified PCR products on the *E. coli* 16S rRNA gene as the template. Each reaction was performed in the presence of 0, 1, 10, 100, and 1,000 μM aminoglycoside.

methyltransferases of aminoglycoside-producing actinomycetes. This suggested that NpmA would also modify the same position within 16S rRNA, as reported previously (3, 22, 43). To determine the exact position of methylated nucleotide, a hybridization protection study was first carried out with deoxynucleotides that were complementary to a part of the 16S rRNA sequence. Two oligomers from positions 1392 to 1421 and positions 1478 to 1507 were prepared to span the aminoglycoside-binding A-site region within the 16S rRNA. The hybridization with the oligomer from positions 1392 to 1421 served to keep the radioactivity of [^{3}H]methyl-labeled 16S rRNA after RNase T_1 digestion, while the oligomer from positions 1478 to 1507 was ineffective in protecting against RNase T_1 digestion (Fig. 4A). This finding indicated that the position of the methylated nucleotide is located within the region from residue 1392 to residue 1421 in the 16S rRNA.

Primer extension. Methylated 16S rRNA, prepared from 30S subunits which were incubated with His_5-NpmA in the presence of the methyl donor SAM, was used as the template RNA in reverse transcriptase extension. The extension terminated at position C1409, indicating that methylation surely occurs at position A1408 (Fig. 4B). In contrast, no termination signal was observed at the same position when unmethylated 16S rRNA was used for the reverse transcription experiment (Fig. 4B).

HPLC assay of methylated adenine residue. We determined the type of detailed modification by HPLC. When wild-type 16S rRNA was treated with nuclease P1 plus alkaline phosphatase, there was no peak corresponding to 1-methyladenosine (m^1A), due to the lack of an innate m^1A nucleoside in the 16S rRNA of a K-12-derived *E. coli* strain (Fig. 5A). On the other hand, the m^1A peak was clearly observed when the 16S rRNA methylated by NpmA was analyzed (Fig. 5B). These results clearly demonstrate that NpmA actually methylates the N-1 position of adenosine. Thus, NpmA is an adenine N-1 methyltransferase. Each peak corresponding to m^5C, m^7G, m^3U, and m^2G was detected with almost equal intensity between the wild-type and the methylated 16S rRNAs.

Binding of aminoglycosides to 30S subunits assayed by footprinting. The interaction between the 30S subunit and aminoglycosides was monitored by determining the changes in the chemical modification pattern of 16S rRNA by using DMS. The RNA footprints at G1494 were analyzed by primer extension with reverse transcriptase (Fig. 6). The apparent protection of G1494 was observed when wild-type 30S subunits were treated with gentamicin, neomycin, and ribostamycin at concentrations of 100 μM and 1,000 μM, indicating the certain binding of aminoglycosides to the 30S subunits. On the other hand, no decrease in the signal on G1494 was observed at 100 μM and 1,000 μM when m^1A1408-methylated 30S subunits were used under the same reaction conditions, indicating the interruption of aminoglycoside binding by methylation at A1408. Although a slight increase in the signal at position G1494 in the m^1A1408-methylated 30S subunits was observed in a gentamicin concentration-dependent manner, the precise reason for the phenomenon remains uncertain from the findings of the present study.

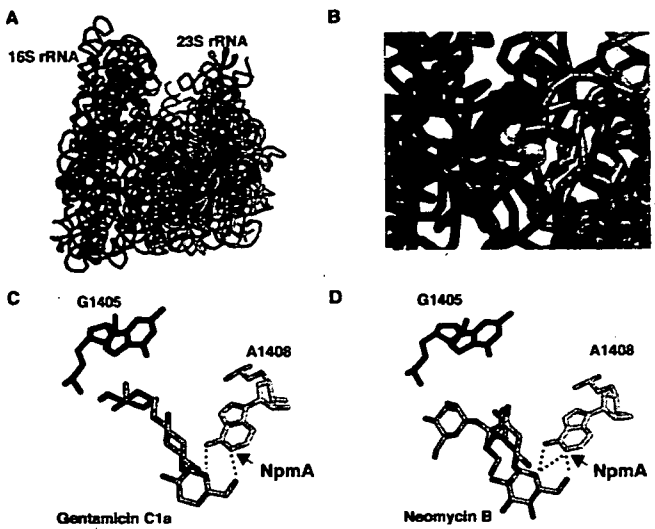

FIG. 7. Predicted interaction between aminoglycosides and 16S rRNA in the 30S ribosomal subunit. (A) Complex structure of 16S rRNA (black) and 23S rRNA (orange) in the 70S ribosome from *E. coli* (PDB codes 2AVY and 2AW4) (41). The positions of G1405 (magenta) and A1408 (cyan) are indicated with dots. (B) Enlargement of the region surrounding G1405 and A1408 at the same angle used for panel A. (C and D) Three-dimensional model from the crystal structure of complexes between aminoglycosides (C; gentamicin C1a, PDB code 2ET3) (D; neomycin B, PDB code 2ET4) and nucleotides G1405 and A1408 in the decoding A site (15). The dashed lines indicate probable hydrogen bonds. The figures were rendered with the PyMol program.

DISCUSSION

A careful review of the recent literature enabled us to understand the binding mode between aminoglycosides and the target site through the resolution structure investigated by X-ray crystallography and nuclear magnetic resonance imaging (6, 13–15, 29, 38, 39, 40, 54). Basically, aminoglycosides such as 4,5- and 4,6-disubstituted 2-deoxystreptamines form specific hydrogen bonds with the nucleotides in decoding region A-site within 16S rRNA (Fig. 4C and Fig. 7C and D). Thus, mutations and modifications in the key nucleotides that perturb the hydrogen bond result in the loss of binding between aminoglycosides and 16S rRNA and lead to resistance to aminoglycosides in bacteria. The production of methyltransferase, which converts guanosine to 7-methylguanosine (m^7G) at position 1405 in 16S rRNA, is one representative mechanism of aminoglycoside resistance in the manner described above. The genes encoding guanine N-7 methyltransferases, which methylate position G1405, have been found on the chromosomes of aminoglycoside-producing actinomycetes and on the plasmids of various pathogenic gram-negative pathogens isolated in both clinical and veterinary settings (7, 10, 11, 17, 20, 34, 48, 53). Furthermore, it is well known that adenine N-1 methyltransferases which modify position A1408 belong to another group of 16S rRNA methyltransferases that confer resistance to multiple aminoglycosides. However, this has so far been found exclusively on the chromosomes of aminoglycoside-producing actinomycetes. The present study is the first to describe the emergence of plasmid-mediated adenine N-1 methyltransferase, which confers panaminoglycoside resistance among pathogenic bacteria.

Position A1408 plays a crucial role in the binding of 4,6- and 4,5-disubstituted 2-deoxystreptamines, because the puckered sugar ring I of these agents is inserted into the A-site helix to form hydrogen bonds to Watson-Crick sites N-1 and N-6 of the universally conserved A1408 among bacteria (Fig. 7C and D) (6, 15, 46). Thus, alternation of A1408 to G leads to a repulsive interaction with the 6′-NH_3 group at ring I of 2-deoxystreptamines, while 2-deoxystreptamines carrying a 6′-OH group can still interact with 16S rRNA by accepting a hydrogen bond from the N-1 and N-2 positions of G1408 (35, 46). Likewise, methylation at the N-1 position of A1408 by NpmA will disturb the formation of the hydrogen bond toward the N-6′ or O-6′ of ring I of aminoglycosides, and this would, in turn, reduce the binding affinities of aminoglycosides to the target. In fact, the NpmA-producing strains demonstrate resistance to various aminoglycosides that combine the N-1 of A1408 through ring I. On the other hand, NpmA production did not confer resistance to non-A-site binders, such as streptomycin and spectinomycin. The results of susceptibility testing are well consistent with their footprinting patterns, with protection against DMS modification at G1494.

Additionally, it is speculated that m^1A1408 methylation will fundamentally affect the formation of the A1408 · A1493 base pair pocket, which is essential for aminoglycoside binding. However, a dynamic conformational change in RNA structure might impair a number of ribosomal innate functions, including decoding, aminoacyl transfer, and translocation. Actually, A1493 participates in codon-anticodon recognition during the tRNA selection step and involves a conformational change from a "tucked-in" form to a "flipped-out" form (28, 31). Although the effect of m^1A1408 methylation on the innate rRNA function remains uncertain, it seems unlikely that m^1A1408 methylation would be a serious disadvantage for bacterial proliferation, because there is no significant difference in the doubling times between NpmA-producing *E. coli* and wild-type *E. coli* strains under culture conditions with both rich and minimal medium compositions (data not shown). A growth competition assay may be required to elucidate the accurate biological cost induced by m^1A1408 methylation in bacteria.

The methylation reaction by an innate C1407 16S rRNA methyltransferase, YebU, of *E. coli* is specific for the 30S subunits and not for the naked 16S rRNA molecule (1). Docking of YebU onto the 30S subunit revealed several contacts between the methyltransferase domain of YebU and ribosomal protein S12 as well as 16S rRNA (21). Hallberg et al. concluded that interactions of YebU with ribosomal protein would explain the substrate specificity seen in YebU (21). Obviously, the accessibility of YebU to the 30S subunit would be supported by the fact that the C1407 position is exposed in the 30S subunit as well as in the 16S rRNA. As expected, the substrate specificity of NpmA is similar to that of YebU (Fig. 3B), and the explanation for this specificity might partially be the same reason suggested for YebU (Fig. 7A and B). A similar substrate specificity was also observed in a part of the aminoglycoside-resistant G1405 16S rRNA methyltransferase group (27). Methylation at an exposed position such as A1408 would occur in the late stage, during the assembly of the 30S subunit.

The G+C content of A1408 16S rRNA methyltransferase genes from aminoglycoside-producing actinomycetes is greater than 70%, whereas that of *npmA* is 34%. This discrepancy would make it unlikely that the origin of *npmA* is aminoglycoside producers with high G+C contents. A similar discrepancy was also observed in the case of the G1405 16S rRNA methyltransferases of actinomycetes and pathogenic bacteria. Liou et al. indicated that aminoglycoside producers with low G+C contents, such as *Bacillus circulans*, which naturally produces butirosin, might be the candidate sources of plasmid-mediated 16S rRNA methyltransferases (27). Although questions remain as to the presence of a 16S rRNA methyltransferase that confers aminoglycoside resistance in the genus *Bacillus*, the gene products of a putative ABC transporter substrate binding protein (*orf7*) and a mobilization protein (*orf8*) located at the 3′ end of *npmA* certainly have relatively low levels of identity to those of *Bacillus* spp. The detailed characterization of 16S rRNA methyltransferases in aminoglycoside-producing *Bacillus* spp. demonstrating low G+C contents might provide clues to the identification of the origin of plasmid-mediated 16S rRNA methyltransferases, including *npmA*.

In conclusion, to our knowledge this is the first time that a novel plasmid-mediated m^1A1408 16S rRNA methyltransferase, NpmA, was identified in a panaminoglycoside-resistant *E. coli* clinical isolate. Indeed, methylation at A964 (pactamycin resistance) (2), G1405 (kanamycin-gentamicin resistance), and A1408 (kanamycin-apramycin resistance) and the loss of methylation at G527 (streptomycin resistance) (33), C1409 (capreomycin resistance) (23), and A1518-A1519 (kasugamycin resistance) (45) have been reported so far to be mechanisms of resistance to 30S subunit-targeting drugs in bacteria. However, these mechanisms have not been fully understood, especially in pathogenic bacteria that tend to be continuously or intermittently exposed to various aminoglycosides in both clinical and livestock farming environments. Further study is warranted to clarify the molecular mechanisms underlying the panaminoglycoside resistance that has been acquired by pathogenic bacteria.

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