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特集

Helicobacter Year Book 2010〜2011
—*Helicobacter* はここまで解明された—

Helicobacter 研究の年間レビュー

感染ルートはここまで解明された

大崎敬子*　神谷　茂*

2010年に公表された*Helicobacter pylori*（*H. pylori*）の感染ルートに関する研究を中心にレビューした．家族内感染の原因として，母子感染のほか同胞からの感染の重要性についても複数の報告で示された．また，感染率の高い国では依然として，公衆衛生上の問題点も残っている．口腔内における*H. pylori*の存在として，これまでの唾液や歯垢を材料とする研究のみならず，扁桃や食道組織中で菌の存在が明らかになり，胃外でも持続感染している例が明らかになってきた．これらが胃に*H. pylori*が持続感染している結果に付随する現象かどうかは検討する余地があろうが，つぎのステップとして培養法で生菌が証明されれば，感染源として重要な存在と考えられる．また，バイオフィルムや飲料水からのリアルタイムPCR法や培養による*H. pylori*の存在が明らかにされたことも興味深く，続報が期待される．

KEY WORDS
transmission，*Helicobacter pylori*（*H. pylori*）感染

はじめに

2010年度の掲載論文から，“*H. pylori*”と“transmission”というキーワードで検索するとわずかに34報であった．そのなかから，レビューや英語でない言語の論文を除外すると，感染ルートの証明となる報告は数少ない．感染媒体からの*H. pylori*の培養が困難であること，おもにPCR法をおこなってDNAの存在を調べる方法のみでは生菌の証明とはならないことなどの理由から，新規の論文が掲載されにくい状況にあるものと考えられる．疫学研究のなかから，感染のルートを明らかにする目的で*H. pylori*の陽性率を調べ，感染のリスクファクターを検討している報告を加えて紹介する．

1．感染のリスクファクター

1）家族内感染を中心として

近年，国内の*H. pylori*感染については家族内感染による頻度が最も高いものと考えられている．したがって，ここで紹介する論文は海外のものであっても，国内における*H. pylori*感染予防の観点から参考になると思われる．ただし，ここに紹介する内容とわが国の場合とでは，親の世代の*H. pylori*感染率が大きく異なってきている

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ことは考慮されるべき問題である．

ブラジルの都市部に住んでいて収入が低い集団，128家族570名を対象におこなった *H. pylori* 感染についての調査がある[1]．大人は血清抗体価の検査，子供は尿素呼気試験を実施して，感染の有無を確認した．6歳未満の子供で28.6%，親である40歳以上の成人で82%の感染率であり，年齢とともに感染率の有意な上昇が認められた．また，感染者の母をもつことと，感染した同胞を一人以上もつことはそれぞれ独立したリスクファクターであり，父親の感染は関係しないと報告された．また，イスラエルの子供197名（平均年齢1回目3.7歳，2回目7.8歳）を対象にした調査では，同じ地区で2回の *H. pylori* 検査をおこない，140名が2回の検査を受けた[2]．持続感染69名（49.3%），新規感染14名（10.0%），未感染56名（40.0%）であった．2回目の検査で陰性化したのは1例（0.7%）であり，3～5歳の感染率は49.7%，6～9歳で58.9%であった．*H. pylori* 陽性者と非陽性者のあいだの比較において有意差があったのは，家族全体の収入が低い，家の面積に対する住居者が多い，母親の教育年数が短いなどの特徴と，同胞に感染者がいることであった．これらの要因が *H. pylori* 感染に関与する可能性が指摘された．

1998～2005年までテキサス-メキシコ境界で集められたデータによるPasitosコホート研究がある[3]．その結果，年上の兄姉での慢性持続 *H. pylori* 感染はいつも弟妹の持続感染に先行していた．母親の感染状態，母乳育児，抗菌薬の使用，および社会経済学的因子を調整してもその効果は明らかだった．とくに兄弟間の年齢差3年で最も関連性が高かった．

母子感染の頻度および乳児の抗体価の変動について明らかにする目的で，2歳までの乳幼児に，3ヵ月ごとに調査をおこなったバングラデシュからの報告がある[4]．group Aは0～12ヵ月までに便中抗原測定法が陽性，group Bは12～24ヵ月で陽性，group Cは全期間陰性の群とした．母親由来の抗体は生後0～6ヵ月にかけて減少した．6ヵ月後の抗体価（IgG）をgroup間で比較したところ，group Bは他のグループとくらべて有意に高く，生後3ヵ月での抗 *H. pylori* IgA抗体についても3群間で最も高かった．この報告によると乳児への *H. pylori* の感染は生後3～6ヵ月以降に増加するものと考えられる．

2）公衆衛生の問題

ここに紹介するリスクファクターは，現在のわが国では改善されてきていると考えられる．

ブラジルの北東部Salvadorでおこなわれた調査では公衆衛生の不十分な状態が *H. pylori* の感染に関連していることが示された[5]．オッズ比の高い要因としては，年齢が8歳以上，同胞の数の多さなどがあるが，ほかに，保育所の通所歴があること，道路が舗装されていない地区内に住居があること，トイレが水洗でないことなどの公衆衛生上の問題が指摘された．ウガンダでは0～12歳の子供427人を対象として，便中抗原測定法（HpSA）と血清抗体（Immnocard）法の併用による *H. pylori* の感染調査がおこなわれた[6]．1歳未満は28.7%，1～3歳ですでに46%となり，それより上の年齢でも40%以上の感染率を示している．また，女児よりも男児の感染率が高く（38.5%と49.8%），住居のタイプが定住住宅であるかどうか，水洗トイレの有無によって感染率に有意な差があった．

3）職業上のリスクファクター

ベルギーのフランダース地方の汚水処理施設で働く317人について血清抗 *H. pylori* 抗体価を調べ，製薬会社社員の250人と比較した結果が報告された[7]．汚水処理施設労働者および製薬会社の社員の *H. pylori* 感染率はそれぞれ16.7%および13.6%であり，両群間に有意差は認められなかった．汚水処理場で感染が起きるという考えは，2002年のOrsiniら[8]の報告で，汚水から発生するエアロゾルに *H. pylori* 遺伝子が検出されたことに由来する．しかし，この調査だけでなく，他の調査でも汚水処理施設の労働者に有意に高い感染率は認められてはいないため，処理場内で感染が起きる頻度はきわめて低いものと考えられる．

海水利用区（Paikgacha, Khulna）と真水利用区（Derai, Sunamgonj, Dhaka）でFish Handler（漁業水産業従事者）に対して調査を実施した報告がある[9]．Fish Handlerに

おける *H. pylori* 感染率は同地区に暮らす他の職業従事者の対照群と比較して有意に高かった．血清抗体価の陽性者は Fish Handler で 77.3%に対して，対照群は 37.5%である．しかし，海水利用区と真水利用区のあいだの *H. pylori* 感染率に有意差を認めていないので，*H. pylori* 感染が海水由来もしくは真水由来で起こるのかは不明である．Fish Handler で感染率が高くなる原因として，魚の棲む水がヒト糞便で汚染されていることが考えられるが，魚の体内に *H. pylori* が持続感染しているという報告はこれまでない．ただし，海水から *H. pylori* が検出された報告はあるので，今後原因を解明する続報が期待される．

2．口腔内の *H. pylori* の存在

Kusano ら[10]は扁桃での *H. pylori* 感染率について検討を加えた．再発性扁桃炎と IgA 腎症で，扁桃摘出術を施行された患者 55 名を対象として，扁桃陰窩から *H. pylori* coccoid（球状化菌）が 43 名に検出された．ELISA 法と尿素呼気試験の結果からそのうち 15 例で胃に *H. pylori* が感染していることが明らかになった．しかし，扁桃に *H. pylori* が存在している全員が胃に *H. pylori* をもっているわけではなかった．*H. pylori* 陽性の 43 例中 17 例で免疫蛍光染色により *H. pylori* 菌体が扁桃陰窩に認められ，菌体内部には CagA 抗原が示された．すべて coccoid として存在していて，鞭毛はもたなかった．Cellini ら[11][12]は唾液と食道からの生検サンプルを対象として *H. pylori* の検出をおこなった．食道生検サンプルは臨床上貴重なサンプルであり，胃内に *H. pylori* が感染している 19 例から採取された．生検材料の免疫組織染色により 19 例中 3 例 15.8%に[11]，さらに nested-PCR 法によりすべての検体（100%）に *H. pylori* を検出し，PCR 産物の塩基配列を決定することにより[12] *H. pylori* を同定している．唾液や食道に存在する本菌が感染源となることが考察された．

Wnuk ら[13]が唾液を用いて PCR 法をおこなった研究では，102 例の唾液中，*cagA*$^+$/*ureA*$^+$が 42 例，*ureA*$^+$/*cagA*$^-$が 24 例みつかった．さらにそれらの唾液の抗酸化活性を測定して，*H. pylori* の産生物が酸化ストレスの誘導にどのようにかかわるのかを研究した結果，*cagA*$^+$/*ureA*$^+$または *ureA*$^+$/*cagA*$^-$の例で，*cagA*$^-$/*ureA*$^-$とくらべて総抗酸化活性は低く抑えられているのが明らかになった．さらに *cagA*$^+$/*ureA*$^+$と *ureA*$^+$/*cagA*$^-$の唾液の比較では，*cagA*$^+$/*ureA*$^+$でより低い総抗酸化活性が示され，口腔領域でも CagA 蛋白が抗酸化活性に影響すると報告されたことは大変興味深い．

ポーランドでは，155 人の患者から歯垢を回収し，便中抗原測定キットを使って *H. pylori* 感染が調べられた．その結果，65.6%が陽性であった[14]．*H. pylori* 陽性・陰性の患者で，歯の健康状態については差を認めなかった．30 人の唾液，歯垢および胃粘膜を対象として *H. pylori* の各遺伝子が PCR 法で検出された[15]．胃粘膜材料の 80%，唾液 20%，歯垢 30%で *ureA*，16SrDNA の遺伝子増幅を認めた．さらに，*vacA* 遺伝子型をシークエンスによって調べた結果，特定の *H. pylori* が患者の口腔と胃に感染していることが示され，同一の菌株に感染していることが明らかになった．口腔領域から *H. pylori* は高頻度に検出されるが，検出方法が PCR 法だけにもとづく場合，その結果は慎重に解釈されるべきである．

Vilarinho ら[16]は，咽頭扁桃切除および口蓋扁桃切除術が施行された 62 人の子供を対象として，101 例の外科標本（55 例の咽頭扁桃と 46 例の口蓋扁桃）を集め *H. pylori* の検出をおこなった．*H. pylori* 血清抗体価および切除組織を用いて迅速ウレアーゼ試験，免疫組織染色，fluorescent *in situ* hybridization（FISH）法および PCR-DNA ハイブリダイゼーション解析（PCR-DEIA）などの検査がおこなわれた．62 人中 24 人に抗 *H. pylori* 抗体が示され，そのうち 3 人が扁桃組織の迅速ウレアーゼ試験は陽性であった．免疫染色で陽性の患者 2 人は，血清抗体価は陰性であった．迅速ウレアーゼ試験か免疫組織染色で陽性例に FISH 法をおこなった結果，すべて陰性であった．PCR-DEIA 法ではテストした全サンプルが陰性であった．本報告からは扁桃組織に感染源となるような *H. pylori* の存在は否定的な結果となった．現在胃の *H. pylori* を検出するのに使用されているテクニックだけでは，咽頭扁桃組織の感染を評価するには適切ではないのかもしれない．

以上のように，口腔内から *H. pylori* が検出されるのは

もはや間違いないが，それが果たして感染源となるのかという疑問を解決するにはやはり培養によって菌を証明することが必要だと考える．

3．糞-口感染

A 型肝炎ウイルス HAV は糞便中に排泄され，糞-口感染が主たる感染経路である．トルコのアナトリア地方における，母子感染の状況を調べた論文では，母子 48 組に対して *H. pylori* 抗体価と，HAV に対する抗体価の保有状況が調べられた[17]．出生時の調査で母乳中および血清中（母子とも）にきわめて高い抗 *H. pylori* 抗体の存在（81.3%および 95.8%）が示された．子供の血清や母乳中の抗体価は 9 ヵ月後まで減少し，陽性率が 39.6%となり，12 ヵ月後にふたたび上昇した（陽性率 47.9%）．新規感染者が少なくとも 4 例は含まれた．抗 HAV 抗体は抗 *H. pylori* 抗体の保有と非常によく似た状況であることから，両者は同じ感染ルートをもつことが示唆される．

同じく，サウジアラビアでは，1,200 名の 16～18 歳の学生を対象として，抗 *H. pylori*-IgG と抗 HAV-IgG が調べられた．*H. pylori* の感染率は 47%であった[18]．しかし，クロス集計の結果では，両方の抗体価をもつものはわずか 8%であった．抗 *H. pylori*-IgG と抗 HAV-IgG のあいだには有意な相関はないものと結論づけられた．

相反する報告が 2 報つづいたが，これには国や地域の公衆衛生状態が反映されるため，どちらが正しいと一概に述べることはできない．わが国でも 1990 年代のおわりに両者の関連についての研究が報告されたが，感染率が少なくなると不一致例が多くなってくることから近年の報告はない．*H. pylori* の場合，糞便で汚染された食べ物などから本菌は分離されていないことから，少なくとも HAV 感染と結びつけるデータは不足している．

4．水系感染

ドイツの Leipzig にある二つの農村部でおこなわれた調査において，*H. pylori* 感染と有意な正の相関があったのは，1 世帯に 3 人以上の子供が暮らすこと，平均以上の家族の密度で暮らすこと，大通りに面した家に住むことおよび井戸水の使用であった[19]．また，感染予防に働く因子としては，「感染率の低い地域で暮らすこと」および「ドイツ生まれであること」が明らかにされた．これらの結果から，著者らは長期間にわたる，大気や水の汚染が本菌の感染源となる可能性を示唆した．

2006 年 10 月～2007 年 7 月のあいだに Basra governorate の 22 の地区から，飲料水の合計 198 個のサンプルが集められ，細菌の分離培養がおこなわれた．変法コロンビア尿素寒天で，469 コロニーの細菌が培養され，173 コロニーが同定された．このうち 14 コロニーだけが *Helicobacter* 属菌と同定され，10 コロニーが *H. pylori* であると同定された．この細菌は 0.5 mg/*l* の塩素濃度に耐性を示した．これは飲料水における *H. pylori* の分離の最初のレポートだとして報告された[20]．

H. pylori は，環境が悪くなったときに，生きてはいるが培養が不可能な状態（vaiable but non culturable condition：VBNC）に入る能力がある．VBNC を検出するための新しい解析方法が必要である．飲料水材料を使用してバイオフィルムを作製し，VBNC を早く確実に検出する方法として *ureA* サブユニットをターゲットとした定量的リアルタイム PCR 法が確立された[21]．この方法で，水にできたバイオフィルムから 10 コピーの *H. pylori* を検出することが可能となった．飲料水バイオフィルムはシリコンチューブモデルで作製したもので，菌がスパイクされたサンプルとされないサンプルの両方に *H. pylori ureA* 遺伝子を認めた．本論文は検出方法の確立にとどまっているが，今後応用され，さまざまな場所のサンプルより VBNC が検出できるようになることが望ましい．

おわりに

2010 年の 12 月に Epub で読むことのできた論文がある[22]．環境水から *H. pylori* の 16SrRNA を DNA のみならず RNA で検出している論文である．DNA だけのデータとくらべて，この菌が生菌であるということの証拠になる．21%のサンプルから DNA が検出でき，そのうち 80%から RNA も検出できていることから，環境水中に *H. pylori* が生菌の形で存在していることが想定される．今後の研究調査の手法として応用されることが期待される．

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Attenuated CagA Oncoprotein in *Helicobacter pylori* from Amerindians in Peruvian Amazon*[S]

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Population genetic analyses of bacterial genes whose products interact with host tissues can give new understanding of infection and disease processes. Here we show that strains of the genetically diverse gastric pathogen *Helicobacter pylori* from Amerindians from the remote Peruvian Amazon contain novel alleles of *cagA*, a major virulence gene, and reveal distinctive properties of their encoded CagA proteins. CagA is injected into the gastric epithelium where it hijacks pleiotropic signaling pathways, helps Hp exploit its special gastric mucosal niche, and affects the risk that infection will result in overt gastroduodenal diseases including gastric cancer. The Amerindian CagA proteins contain unusual but functional tyrosine phosphorylation motifs and attenuated CRPIA motifs, which affect gastric epithelial proliferation, inflammation, and bacterial pathogenesis. Amerindian CagA proteins induced less production of IL-8 and cancer-associated Mucin 2 than did those of prototype Western or East Asian strains and behaved as dominant negative inhibitors of action of prototype CagA during mixed infection of Mongolian gerbils. We suggest that Amerindian *cagA* is of relatively low virulence, that this may have been selected in ancestral strains during infection of the people who migrated from Asia into the Americas many thousands of years ago, and that such attenuated CagA proteins could be useful therapeutically.

Population and molecular genetic studies of genes of bacterial pathogens whose products interact with host tissues can give important insights into evolutionary forces, enhance understanding of infection and disease processes, and potentially lead to improvements in human health. *Helicobacter pylori* (Hp)[3] chronically infects the gastric epithelial surface and a narrow band of overlying mucus in billions of people worldwide (1–3). Most Hp infections begin in infancy and can last for decades despite features such as inflammatory responses, mucosal shedding, and peristalsis that make the gastric mucosal niche hostile to nearly all other microbes. Hp constitutes a major risk factor for several gastroduodenal diseases including peptic ulcer and gastric cancer even though most Hp infections are asymptomatic.

DNA sequence analysis of representative housekeeping genes (multilocus sequence typing) has shown that Hp is extremely diverse genetically and that different genotypes predominate in different geographic regions or human populations (4–7). Hp prevalence and multilocus sequence typing studies have indicated that Hp transmission occurs preferentially within families and local communities, not in sweeping epidemics. Such localized transmission fosters accumulation of genetic diversity by genetic drift and selection for adaptation to local conditions including differences in human physiology. Multilocus sequence typing analyses have indicated that probably Hp has infected humans for eons and was carried by the people who migrated into Eurasia from Africa some 60,000 years ago and by the ancestors of modern Amerindians who migrated from Asia into Beringia and then into the Americas some 20,000 years ago (4–7). Geographic differences among alleles of several genes whose products interact with host tissues (such as *cagA*, *vacA*, and *babA2*) are even greater than those of housekeeping genes (4–6, 8). Much of this extra diver-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1–S9.

The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AB587140–AB587258.

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[3] The abbreviations used are: Hp, *H. pylori*; CagA, cytotoxin-associated gene A; CRPIA, conserved repeats responsible for phosphorylation-independent activity; W, Western; EA, East Asian; AM, Amerindian; IKK, IκB kinase; MUC2, Mucin 2; NF-κB, nuclear factor-κB; SRE, serum response element; CFU, colony forming unit(s); mAb and pAb, monoclonal and polyclonal antibodies, respectively; PY, phosphotyrosine.

sity may stem from differences in selective forces operating in different human populations and geographic regions (9, 10).

Prominent among the proteins that Hp uses to manage its special gastric mucosal niche is CagA, a major virulence factor that is injected via a type IV secretion system into gastric epithelium, where it hijacks multiple signaling pathways such as mitogen-activated protein kinases (MAPKs) and phosphoinositide 3-kinase (PI3K)/Akt/IκB kinase (IKK) pathways and thereby alters the actin cytoskeleton, intercellular junctions, and cell polarity and proliferation, and induces inflammation (11–14). Of particular interest are two distinct classes of motifs used by CagA for interaction with host proteins: EPIYA (Glu-Pro-Ile-Tyr-Ala) and CRPIA (conserved repeats responsible for phosphorylation-independent activity) motifs (15). EPIYA motifs undergo tyrosine phosphorylation and mediate tyrosine phosphorylation-dependent interactions with host molecules such as Shp2, Grb2, Crk, Csk, phospholipase Cγ, and PI3K p85 (15–21), whereas CRPIA mediates phosphorylation-independent interactions with Par1, Met, and Tak1 (15, 22, 23).

Recent epidemiological studies revealed that some Hp strains from Amerindians in the Amazon tend to be of a novel genotype, distinct from those of Western and East Asian strains (9, 24–27). Here we describe novel alleles of the *cagA* gene in strains from the remote Peruvian Amazon and the distinctive properties of the CagA proteins they encode. These Amerindian strains are unusual in causing only attenuated cell proliferation and inflammation responses, resulting in lower virulence and decreased risk of severe pathologies including gastric cancer both in *in vitro* and *in vivo* infection models. Much of this attenuation was traced to the low potency of their CRIPA motifs. Furthermore, the attenuated Amerindian *cagA* alleles diminished the strongly proinflammatory response provoked by more pathogenic strains during mixed infection of gerbils. We suggest that this CagA attenuation may have been selected during ancestral human migrations and that it might be useful therapeutically.

EXPERIMENTAL PROCEDURES

Clinical Specimens—Hp strains from the Machiguenga-speaking residents of Shimaa village in the remote Peruvian Amazon were cultured under standard microaerobic conditions with informed consent in protocols approved by local and international human studies committees (25). Hp strains from separate Ashaninka-speaking Amazonian villages in the province of Satipo were cultured under the same protocols. Gastric biopsies were fixed in pH 7.2 buffered formalin, paraffin-embedded, sectioned, hematoxylin/eosin-stained, and graded histologically using the Sidney System (25). Strains from Amerindian Lima shantytown residents were cultured similarly. All Peruvian strains used in these analyses were obtained with informed consent. Other strains used here were from the collections of Sasakawa, Berg, Kamiya, and Zou laboratories. The *cagA* sequences were determined by CagTF and CagTR primer-mediated PCR amplifying the PY region from Hp genomic DNAs (28) and then submitted to the DDBJ/EMBL/GenBank™ databases (accession nos. AB587140-AB587258). Although some Amerindian isolates such as Shi470 have a second apparently defective copy of *cagA* inserted between *cag14* and *cag15* in the middle of the *cag* pathogenicity island (PAI) in addition to *cagA* in the usual *cag* PAI right end location (27), our PCR primers were designed only to detect, clone, and analyze the conventionally placed *cagA* gene.

Sequence and Bioinformatic Analysis—Sequence analysis was performed with programs and data in Blast homology search programs (www.ncbi.nlm.nih.gov) and genome sequence databases. Multiple Sequence Alignments and Unrooted Trees constructed by Neighbor-Joining were performed with programs in ClustalW. Sequence logos analysis was performed with the program in WebLogo 3.

Hp Infection in Cell Culture—Reference Hp strains such as 26695 and ATCC43504 (also often referred to as NCTC11637) and their isogenic Δ*cagA* and Δ*virD4* mutants were previously described (15, 16, 29). ATCC43504 Δ*cagA* derivatives containing chromosomal FLAG-tagged *cagA* (W, W/EA, W/AM-I, or W/AM-II) genes were constructed for this study. Briefly, FLAG-tagged *cagA* genes were ligated with kanamycin-resistant genes as part of the same transcription unit, and then these fragments were ligated between 1000-bp segments that flank the 5′ and 3′ ends of the ATCC43504 *cagA* gene. These DNA constructs were then used for transformation of Δ*cagA* strains with selection for kanamycin resistance and thereby chromosomal placement of our engineered *cagA* genes. Hp strains were cultured according to standard procedures (15, 16). AGS, HEK293, and 293T cells were maintained in Dulbecco's modified Eagle's medium (Sigma) containing 10% fetal bovine serum. Cultured cells were infected with Hp at a multiplicity of infection of 100.

Hp Infection in Cell Culture and Animals—Gerbil infection experiments were approved by the University of Tokyo ethics committee and carried out as described (15, 29). Briefly, 6-week-old male MGS/Sea Mongolian gerbils (CLEA Japan Inc.) were intragastrically inoculated with 10^9 colony forming units (CFU) of Hp strain ATCC43504 or its derivatives. After 8 weeks, the stomachs were examined to determine bacterial load and analyzed by immunohistology. A competition assay was performed by co-inoculating wild-type ATCC43504 and derivatives with engineered *cagA* alleles (10^9 CFU of each strain). The competitive index was calculated as the ratio of the wild-type strain CFU:mutant strain CFU. The two genotypes were distinguished by colony PCR of PY regions in *cagA*. If no CFU were recovered from the stomach, a CFU count of one at the lowest dilution was assigned. Data were analyzed statistically using a Mann-Whitney *U* test for unpaired groups.

Plasmid Construction and Transfection—NF-κB luciferase reporter was from Promega and serum response element (SRE) and p53 luciferase reporters were from Stratagene. Myc-Par1 and Tpr-Met (dominant-active Met) plasmids were kindly provided by Drs. Miki (Osaka University) (30) and Park (McGill University), respectively; the K1110A-Tpr-Met mutant has been described (15). The PY regions of the *cagA* genes from the W (26695), EA (JpnTK1003), AM-I (Shi257), and AM-II (Shi193) strains were cloned into pGEX-6P-1 (Amersham Biosciences) to perform the glutathione *S*-transferase (GST) pull-down assays. The 26695 CagA, ΔPY (Δ871–1026), ΔPY2 (Δ871–976), PR (Y899F/Y918F/Y972F), R952A/R986A, and PR-RA (PR-R952A/R986A) plasmids were previously

described (15). The *cagA* genes from the EA (JpnTK1003), AM-I (Shi257), and AM-II (Shi193) strains were cloned into pEGFP-C1 (Clontech), and their mutants and 26695 ΔPY2 CagA (Δ871–976) mutants were generated using the site-directed mutagenesis kit (Stratagene). The human *IL-8* (−1498/+44) and *MUC2* promoters (−2631/+111) were amplified from genomic DNA of AGS cells and cloned into pGL4.20 (Promega). The 26695 ΔPY *cagA* and Shi193 *cagA* genes were Myc-tagged at their 3′ ends and cloned into pcDNA3.1 (Invitrogen), and the 26695 *cagA* gene was FLAG- or T7-tagged at its 3′ end and cloned into pC4-Fv1E (ARIAD Pharmaceuticals, Inc.) for Fv-mediated homodimerization assays. Culture cells were transiently transfected with appropriate plasmids using FuGENE 6 (Roche Applied Science), Lipofectamine 2000, or Lipofectamine LTX (Invitrogen).

RNA Transfection—To knockdown endogenous host genes, cells were transfected for 48 h with appropriate small interfering RNAs (siRNAs, 100 nM) using Lipofectamine RNAiMAX (Invitrogen). The siRNAs were synthesized, purified, and duplexed by RNAi Co., Ltd. The siRNAs for the luciferase (*Luc*) control, *Par1*, *Met*, and *p85* have been described (15).

GST Pulldown Assay—GST pulldown assays were performed as previously described (15, 16) using GST-fused CagA PY region proteins from W (26695), EA (JpnTK1003), AM-I (Shi257), and AM-II (Shi193) strains, which each harbor EPIYA tyrosine phosphorylation motifs. These proteins were purified from *Escherichia coli* expressing an inducible Elk6-tyrosine kinase domain (strain TKB1; Stratagene) according to the manufacturer's instructions.

Immunoprecipitation, Immunoblotting, and Immunostaining—Immunoprecipitation, immunoblotting, and immunostaining were performed using appropriate antibodies as previously described (15, 16). After immunostaining, specimens were examined using a confocal laser-scanning microscope (Carl Zeiss LSM510), and fluorescent images were analyzed using LSM510 Version 3.2 software (Carl Zeiss). Akt kinase assays using purified GSK3β protein were performed using an Akt kinase assay kit (Cell Signaling Technology) according to the manufacturer's instructions. IKK kinase assays using purified GST-IκB protein were performed as described (31).

Antibodies and Reagents—Rabbit anti-CagA, anti-p-CagA, and anti-UreA polyclonal antibodies (pAbs) were previously described (15, 16). Mouse anti-Crk monoclonal antibody (mAb) was from BD Transduction Laboratories. Mouse anti-phospholipase Cγ and anti-Par1 mAbs were from Upstate Biotechnology. Rabbit anti-p- extracellular signal-regulated kinase 1/2 (Erk1/2; Thr-202/Tyr-204), anti-Erk1/2, anti-p-p38 (Thr-180/Tyr-182), anti-p38, anti-p-Akt (Ser-473), anti-Akt, anti-p-Met (Tyr-1349), anti-p-IκB (Ser-32), and anti-p-Src (Try-416) pAbs and mouse anti-Csk, anti-IKKγ, anti-IKKβ, anti-Tyr(P) (PY20), and anti-Myc mAbs were from Cell Signaling Technology. Rabbit anti-Met, anti-Shp2, anti-Grb2, anti-Stat3, and anti-MUC2 pAbs and mouse anti-p85 mAb were from Santa Cruz Biotechnology. Rabbit anti-p-Tau (Ser-262) pAb and mouse anti-Src and anti-Tau pAbs were from Calbiochem. Rabbit anti-GFP pAb was from Medical and Biological Laboratories. Mouse anti-actin mAb was from Chemicon International. Mouse anti-T7 mAb was from Novagen. Mouse anti-FLAG mAbs and *Anguilla anguilla* agglutinin (for staining gastric pit cells) were from Sigma. Rhodamine phalloidin, Alexa Fluor 633 phalloidin, and TO-PRO-3 were from Molecular Probes.

Luciferase Assay—Luciferase assays were performed using the Dual-luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The firefly luciferase levels were measured and normalized to the activity of phRL-TK-derived *Renilla* luciferase. Data were analyzed statistically using Student's *t* test for unpaired groups and expressed as the means ± S.E. of triplicate experiments.

Homodimerization Assay—Homodimerization assays were performed using the Regulated Homodimerization kit (ARIAD Pharmaceuticals, Inc.) according to the manufacturer's instructions. Briefly, 293T cells were co-transfected for 24 h with the Fv-W-CagA-FLAG, Fv-W-CagA-T7, and W/AM-II-CagA-Myc plasmids in the presence or absence of AP20187 (100 nM), a small molecule that induces homodimerization of Fv domain-containing fusion proteins.

RESULTS

Histopathologic examination of gastric biopsy specimens from 39 Hp-infected Amerindian residents of Shimaa, a small village in the remote Peruvian Amazon (25, 27), indicated that most villagers had active gastritis and mild atrophy, and several had intestinal metaplasia. None had peptic ulcer disease or gastric cancer (Fig. 1*C* and supplemental Table S1). We next examined the *cagA* genes and encoded CagA proteins of Hp cultured from these Shimaa villagers. Prior studies had shown that CagA of European and North American (Western (W)) and East Asian (EA) strains differed markedly in their C-terminal region (PY region) and that these differences are functionally important (11–14). PY regions contain several EPIYA tyrosine phosphorylation motifs: EPIYA-A and EPIYA-B motifs characteristic of both W and EA strains and EPIYA-C and EPIYA-D motifs characteristic of W and of EA strains, respectively (Fig. 1*A*) (11, 14). PY regions also contain CRPIA motifs, which are responsible for separate phosphorylation-independent activities of CagA (Fig. 1*A*) (15).

By sequencing, we found that CagA proteins from most strains from urban Peruvians are of the W type (Fig. 1*A* and supplemental Fig. S1). In contrast, those of Shimaa strains are distinct from both W and EA types and could be placed in two main groups designated AM-I and AM-II (AM, Amerindian) (Fig. 1*B*). Although the overall sequences of AM and W CagAs are similar, AM CagAs have altered or degenerate "EPIYA-B" motifs: ESIYT and GSIYD in AM-I and AM-II CagAs, respectively (Fig. 1*A*). In addition, CagA sequences in some nominally AM-II strains such as Shi30, Shi35, and Shi156 have AM-I type CRPIA motifs (Fig. 1*A*), suggesting that they are actually hybrids and thus might differ functionally from purely AM-I or AM-II type CagA proteins. Most CagA sequences found in strains from the distinct Peruvian Amazonian region of Satipo, which is far from Shimaa and whose residents are of another language group, also fit into this AM-I and AM-II classification, as did CagA of several isolates from Colombian and Venezuelan Amazon (supplemental Fig. S1) (9, 24, 26).

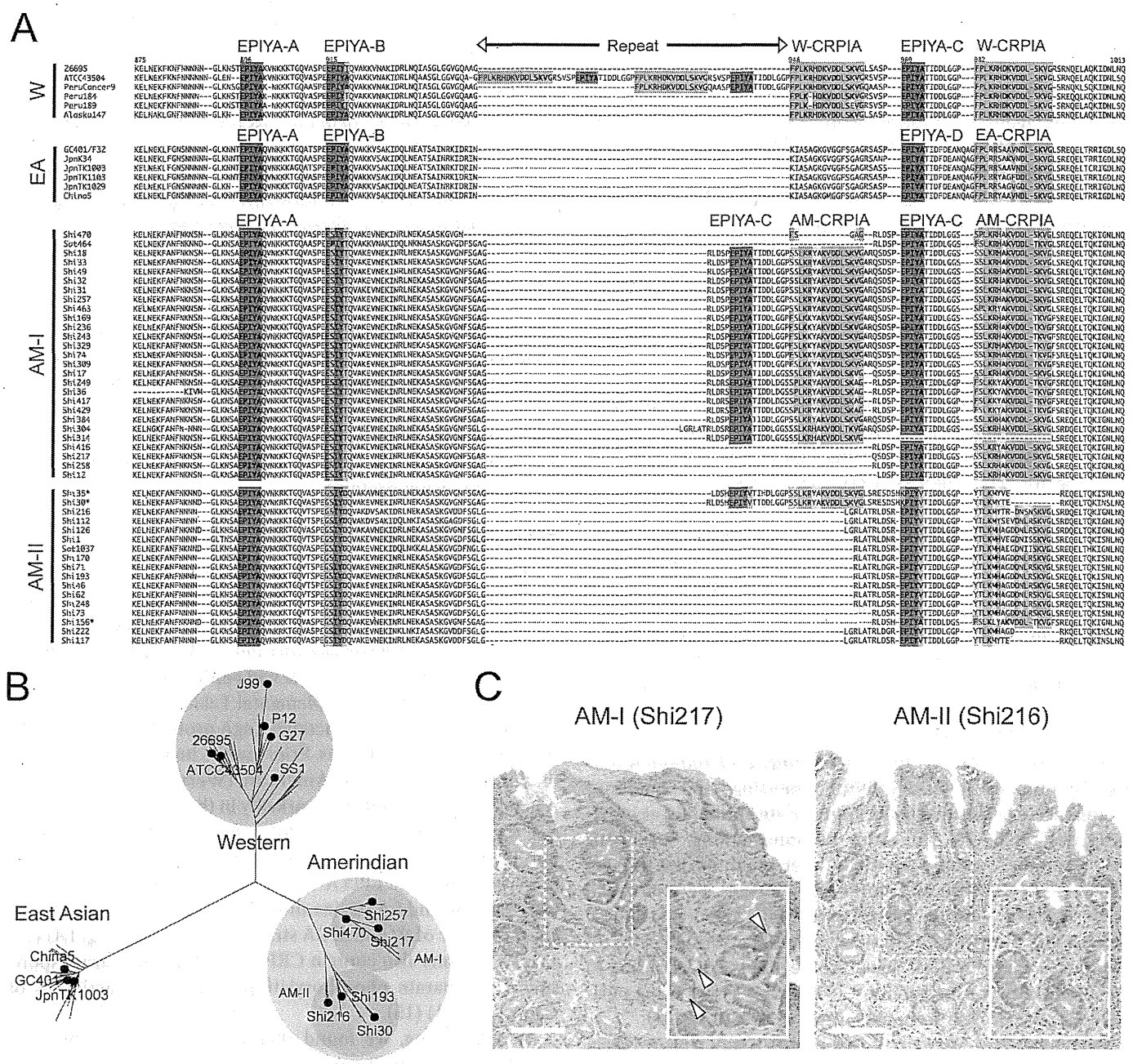

FIGURE 1. **Amerindian CagA is distinct from Western and East Asian CagA.** *A*, shown is multiple sequence alignment of the PY regions of CagA from Hp isolates. The EPIYA (*red*) and CRPIA motifs (*green*) are *highlighted*. CagA was categorized Western (*W*), East Asian (*EA*), and Amerindian (*AM-I* and *AM-II*) based on ClustalW analysis. Amerindian isolates, such as Shi35, Shi30, and Shi156, had marginal CRPIA genotypes between AM-I and AM-II (shown with *asterisks*). *B*, shown is a neighbor-joining tree of amino acid sequences of the PY regions. *C*, shown is hematoxylin/eosin staining of gastric antral biopsies from the indicated Hp-infected individuals. *Bar* = 100 μm. *Arrowheads* indicate goblet cells in sites of intestinal metaplasia.

The CagA N terminus, which is far from its PY region, is needed for efficient CagA translocation into target epithelial cells, subsequent plasma membrane anchoring, and modulation of C-terminal region activities (32–34). The N-terminal regions of each of four AM-I CagAs that we characterized were similar to those of prototype CagA, whereas each of three AM-II CagAs lacked two large internal segments totaling 180 amino acids (supplemental Fig. S2) and thus may well differ functionally from other CagA proteins.

To begin functional testing of AM CagA proteins, we examined CagA tyrosine phosphorylation during Hp infection of AGS cells. CagA from representative W, EA, AM-I, and AM-II strains each underwent such phosphorylation (Fig. 2*A* and supplemental Fig. S3*A*). However, AM-II strains were less effective than W and EA strains in inducing subsequent phosphorylation of the host Erk/MAPK, Akt, and Met and production of IL-8 and cell scattering. AM-I strains, although more potent than AM-II strains, were also less active than W and EA strains in these activities (Fig. 2*B*, supplemental Fig. S3, *B* and *C*), each of which is involved in Hp pathophysiology and associated diseases (15).

CagA-host cell interactions were further examined in several ways. First, GST pulldown assays indicated that PY regions of W, EA, and AM-I CagAs interacted with known CagA-target

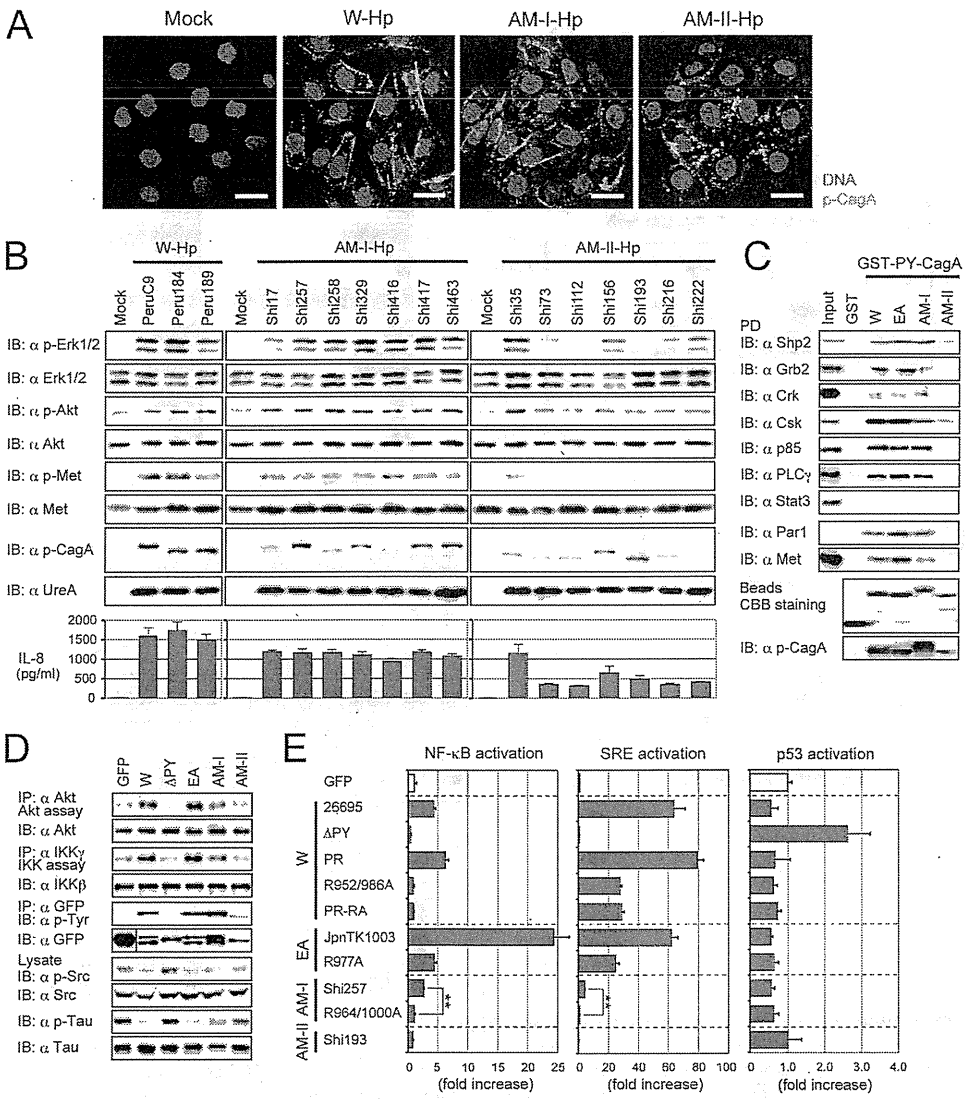

FIGURE 2. **Amerindian CagA has a reduced ability to induce host responses.** *A*, shown is immunostaining of AGS cells infected for 6 h with the indicated Hp strains (*W*, 26695; *AM-I*, Shi257; *AM-II*, Shi193). *Bar* = 20 μm. *B*, immunoblotting (*IB*) is shown of lysates 4 h post-infection (*upper*) and ELISAs for IL-8 in the culture supernatants 8 h post-infection (*lower*). *C*, shown is immunoblotting of lysates (*Input*) and pulled-down proteins (*PD*) by the indicated GST-fused PY regions of CagA (W, 26695; EA, JpnTK1003; AM-I, Shi257; AM-II, Shi193). Coomassie brilliant blue (*CBB*) stains of the purified GST-proteins (*lower*) are shown. *D*, shown are kinase assays and immunoblotting of lysates from 293T cells transfected with the indicated GFP-CagA (W, 26695; ΔPY, 26695 ΔPY; EA, JpnTK1003; AM-I, Shi257; AM-II, Shi193) plasmids and then subjected to immunoprecipitation (*IP*). *E*, shown are luciferase assays on lysates from HEK293 cells co-transfected with the indicated reporters and GFP-CagA plasmids (**, $p < 0.01$). 26695, R952A/R986A, JpnTK1003, R977A, and Shi257 R964A/R1000A are loss-of-function the CagA mutants of CRPIA motifs.

proteins more strongly than did AM-II CagA (Fig. 2*C*). Second, CagA-p85 and CagA-Met interactions are known to mediate phosphorylation of Akt and IKK kinases (15), and CagA-Csk and CagA-Par1 interactions are known to mediate dephosphorylation of Src and Tau kinases, respectively (17, 23). Ectopic GFP-CagA expression in 293T cells showed that AM CagA was less potent than W or EA CagA and that AM-II CagA was less potent than AM-I CagA in Akt and IKK kinase activation and Src and Tau down-regulation (Fig. 2*D*).

Subcellular localization studies showed that AM-II CagA was distributed diffusely and not strictly localized to the plasma membrane, as were other CagA proteins during Hp infection (Fig. 2*A*) or plasmid transfection (supplemental Fig. S4*A*). A W CagA derivative containing internal deletions matching those of AM-II CagA N-terminal regions (called ΔN) was also distributed diffusely (supplemental Fig. S5*A*). These deletions resulted in attenuation of CagA-dependent NF-κB and SRE activation and cell scattering activities (supplemental Fig. S5*B*), which reflects the importance of the CagA N terminus for plasma membrane localization, PY region-mediated intracellular target activation, and general modulation of CagA activities (32–34).

Several CagA chimaeras were constructed and studied to further test these ideas. The PY region of W CagA was replaced by that of EA, AM-I, or AM-II CagA (chimaeras called W/EA, W/AM-I, and W/AM-II) (supplemental Fig. S5*D*). We found that, although these chimeric CagAs localized to plasma membrane microdomains (supplemental Fig. S5*C*), W/AM-I and W/AM-II CagAs activated NF-κB less effectively than did their W parent or W/EA CagA (supplemental Fig. S5*D*). The CRPIA domain promotes cell proliferation and inflammation independent of CagA phosphorylation. The CRPIA domain fifth

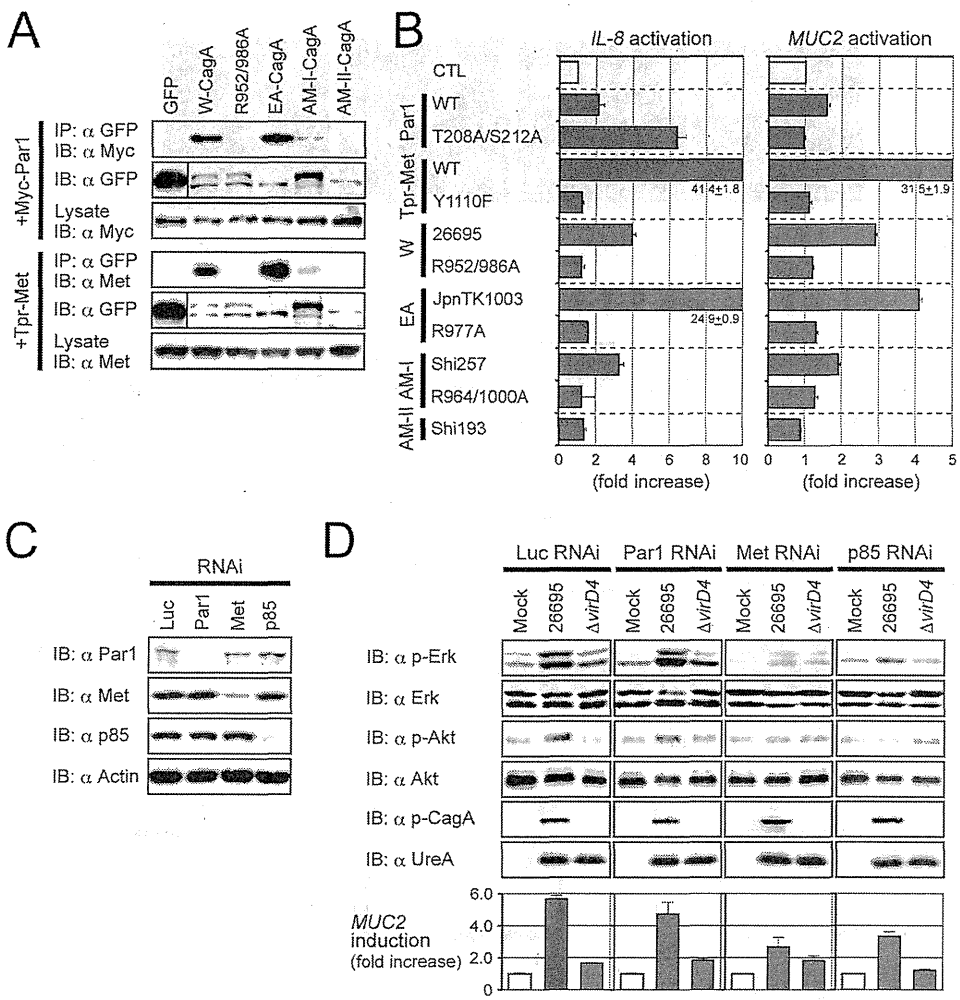

FIGURE 3. **The CRPIA motif is important for CagA-mediated *MUC2* expression.** *A*, immunoblotting (*IB*) is shown of lysates from 293T cells co-transfected with the indicated GFP-CagA (*W*, 26695; *R952/986A*, 26695 R952A/R986A; *EA*, JpnTK1003; *AM-I*, Shi257; *AM-II*, Shi193) and Myc-Par1 or Tpr-Met expression plasmids and then subjected to immunoprecipitation (*IP*). *B*, shown are luciferase assays on lysates from AGS cells co-transfected with the indicated reporters (*IL-8* or *MUC2* promoter) and the indicated GFP-CagA, Myc-Par1 (wild type or kinase-dead T208A/S212A), or Tpr-Met (wild type or kinase-dead Y1110F) plasmids. *C* and *D*, AGS cells were transfected for 48 h with the indicated siRNAs (*C*) and then infected for 4 h with the indicated Hp strain 26695 or its Δ*virD4* derivative (type IV secretion system-defective) (*D*). Immunoblotting of lysates (*C* and *D*, *upper*) and RT-PCR assays for *MUC2* mRNA expression using total RNAs (*D*, *lower*) is shown.

arginine residue is well conserved in W, EA, and AM-I CagA but not AM-II CagA (Fig. 1*A* and supplemental Fig. S1) (15). We generated W, EA, and AM-I CagAs in which this arginine replaced by alanine (W, R952A/R986A; EA, R977A; AM-I, R964A/R1000A). We found these replacement derivatives to be much less able than the corresponding wild-type CagA proteins to activate NF-κB, SRE, T-cell factor/β-catenin, and activator protein-1 (Fig. 2*E* and supplemental Fig. S4*B*). Together these five sets of results indicate that the AM-II CagA protein N-terminal deletion and variant CRPIA sequences each contribute importantly to low potency during Hp infection.

The CRPIA motifs of W and EA CagA are known to interact with Par1 and Met (15, 23). Consistent with the data in Fig. 2*C*, AM-II CagA interacted less with these proteins (Fig. 3*A*) and stimulated less Met-dependent expression of *IL-8* and *MUC2* than did AM-I, W, or EA CagA proteins (Fig. 3*B*). Mucin 2 (MUC2) is a major marker of gastric intestinal metaplasia that itself is a preneoplastic condition closely linked to chronic Hp infection (35, 36). In support of these results, knockdown of endogenous Met or p85 expression (Fig. 3*C*) diminished the ability of Hp to activate Erk and Akt and to stimulate *MUC2* transcription (Fig. 3*D*). We thus interpret that AM CRPIA motifs cause decreased CagA-Met interactions and epithelial cell responses to Hp infection.

Comparison of several W CagA derivatives showed that the strength of NF-κB and SRE activation varied among CagA proteins and that, although W CagA could contain multiple CRPIA motifs, the strength of activation was not strictly proportional to CRPIA copy number (supplemental Fig. S6, *A* and *B*). Given sequence differences among CRPIA motifs of a given type superimposed on consensus motif differences among W, EA, and AM-I and AM-II CagAs (Fig. 4*A*), we hypothesized that these differences in potency stem from differences in CRPIA sequence *per se*, overall PY context, or both.

To test this we constructed derivatives of ΔPY2 CagA (Δ871–976) that contain just one W CRPIA motif (Fig. 4*B* and supplemental Fig. S6*C*) (15) and in which individual residues were changed, often to those of EA CRPIA (supplemental Figs. S6*D* and S7A). In parallel, we also made EA CagA derivatives in which individual CRPIA residues were replaced by residues

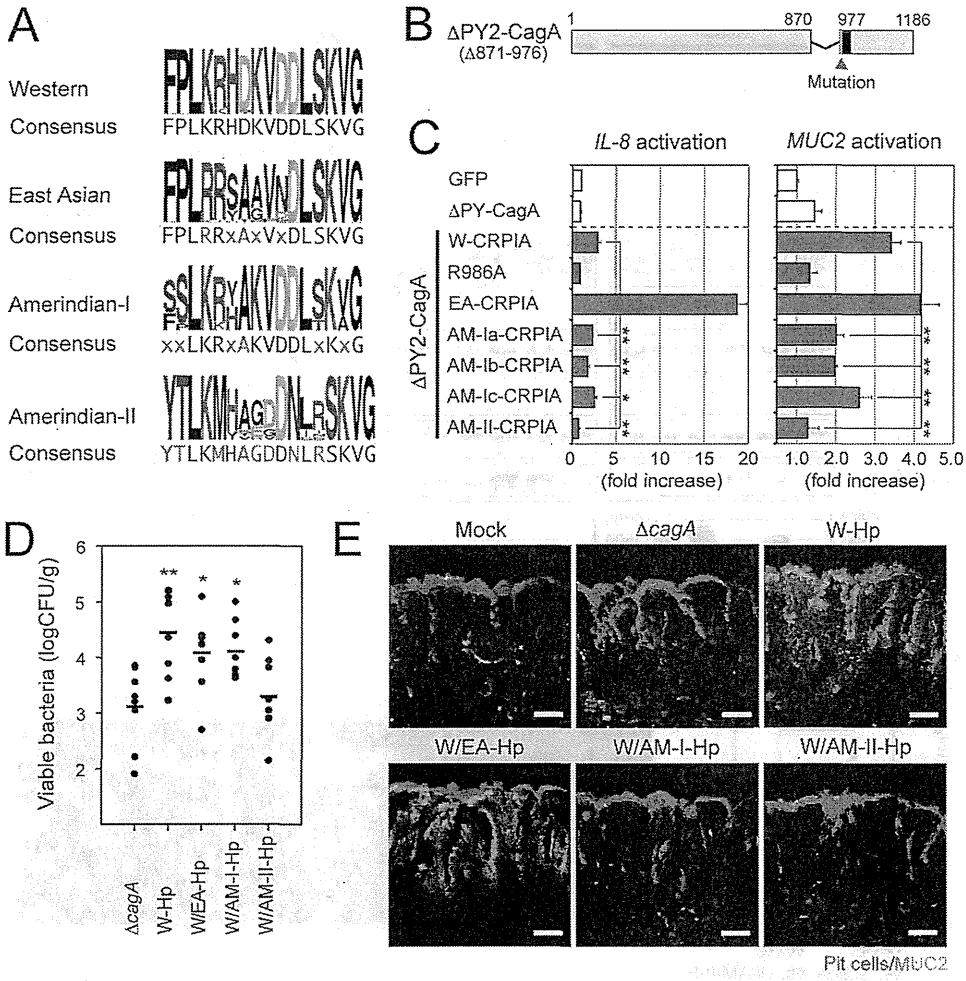

FIGURE 4. **The CRPIA motif in Amerindian CagA is associated with attenuated Hp pathogenesis.** *A*, sequence logos and consensus sequences of the indicated CRPIA motifs of CagA are shown. *B*, schema of ΔPY2 CagA derivative of Western strain 26695 and sites of point mutation changes are shown. *C*, luciferase assays of lysates from AGS cells co-transfected with the indicated reporters (*IL-8* or *MUC2* promoter) and GFP-ΔPY2-CagA plasmids (*, $p < 0.05$; **, $p < 0.01$) are shown. Sequences of W, R986A, AM-Ia, AM-Ib, AM-Ic, and AM-II CRPIA constructs are shown in supplemental Figs. S6–S8. *D*, viable counts of bacteria in the indicated ATCC43504 Hp-infected gerbil stomachs 8 weeks after infection (*, $p < 0.05$; **, $p < 0.01$). *E*, immunostaining of gastric antral sections from infected gerbils. *Bar* = 50 μm.

that are well conserved in W motifs (Fig. supplemental Fig. S7, *B* and *C*). Most single substitutions had only subtle effects on the strength of NF-κB activation (supplemental Fig. S7, *A* and *C*). Stronger effects were obtained by multiple replacements that changed a W CRPIA to an EA CRPIA motif and vice versa (supplemental Fig. S7, *A* and *C*); an ≤2-fold decrease in NF-κB and SRE activation if individual ΔPY2 CRPIA residues were replaced with the corresponding AM-I residues or replacement of W by AM-I motifs (supplemental Fig. S8*A*) but an ~5-fold decrease if the W motif was replaced by an AM-II CRPIA motif (supplemental Fig. S8*B*). These chimaeras also revealed differences between W, EA, and AM CRPIA motifs in strengths of induction of *IL-8* and *MUC2* expression, with AM-II being the weakest of all CRPIA motifs tested (Fig. 4*C*).

Experimental animal infections were used to test if particular CagA types could affect Hp fitness. First, Hp infection studies confirmed that chimeric CagA proteins could be delivered from our engineered Hp strains (containing plasmid-borne TEM-1 β-lactamase tagged, chimeric *cagA* genes (W, W/EA, W/AM-I, and W/AM-II)) into AGS cells (supplemental Fig. S9). In addition, in gerbil infections, strain ATCC43504 derivatives with W, W/EA, and W/AM-I *cagA* genes achieved gastric bacterial titers that were similar to one another and, significantly, ~10-fold higher than those of isogenic strains with Δ*cagA* and W/AM-II alleles (Fig. 4*D*). This implies that W, EA, and AM-I alleles contribute to fitness, whereas an AM-II allele may not. In addition, W and W/EA alleles caused strong gastric epithelial induction of MUC2 expression, whereas W/AM-I caused intermediate, and W/AM-II and Δ*cagA* alleles caused negligible levels of induction (Fig. 4*E*). Thus these experiments indicate that CRPIA motifs of AM, especially AM-II, strains are relatively impotent and further implicate CRPIA as a key determinant of gastric mucosal changes after Hp infection, which affects Hp fitness and disease risk.

Mixed infection experiments provided important additional insight into these issues. W/AM-II Hp infection of AGS cells strongly suppressed the ability of superinfecting W or EA strains to cause Erk, p38, and Akt phosphorylation or IL-8 production, whereas initial infection with an isogenic Δ*cagA* strain did not (Fig. 5*A*). Furthermore, coexpression of W/AM-II and W CagA caused less induction of *IL-8* transcription than did

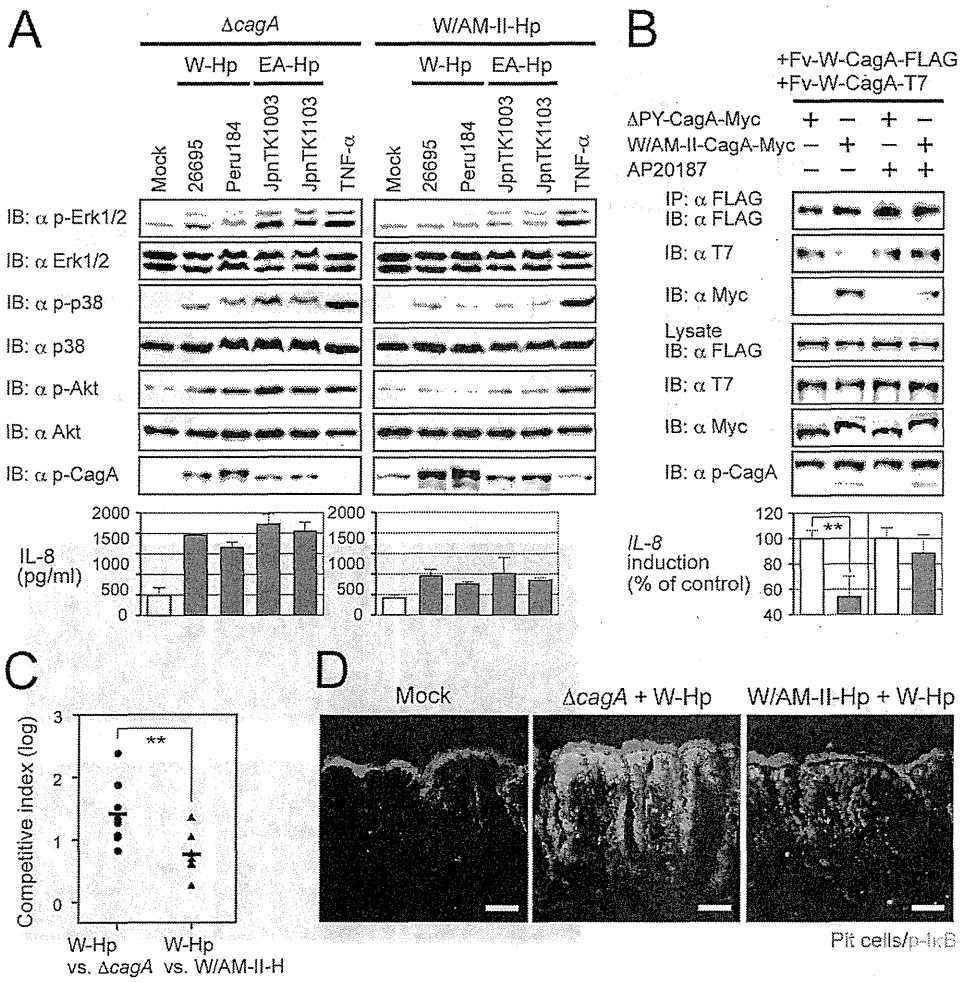

FIGURE 5. **Amerindian CagA competitively inhibits Western and East Asian CagA activities.** *A*, shown is immunoblotting (*IB*) of lysates from AGS cells infected for 2 h with Δ*cagA* or W/AM-II *cagA* containing derivatives of strain ATCC43504 along with W (26695, Peru184) or EA (JpnTK1003, JpnTK1103) Hp or treated with TNF-α (*upper*) and ELISAs for IL-8 in the culture supernatants (*lower*). *B*, immunostaining (*upper*) and luciferase assays (*lower*) are shown on lysates from AGS cells co-transfected with the indicated CagA (ΔPY-CagA-Myc or W/AM-II-CagA-Myc), Fv-W-CagA-FLAG, and Fv-W-CagA-T7 plasmids with or without the dimerizer AP20187 (**, $p < 0.01$). *IP*, immunoprecipitate. *C*, shown is a competitive index of viable counts of bacteria from the stomachs of gerbils co-infected for 8 weeks with the indicated ATCC43504 strains (wild type and Δ*cagA* or wild type and Δ*cagA* complemented with W/AM-II *cagA*) (**, $p < 0.01$). *D*, immunostaining for IκB phosphorylation in gastric antral sections from infected gerbils is shown. *Bar* = 50 μm.

ΔPY and W coexpression (Fig. 5*B*). Because intracellular CagA forms multimers (22, 23), these results suggested interference of W CagA function in mixed complexes that also contained W/AM-II protein.

Indeed, cotransfection studies showed that W/AM-II CagA could form heteromultimers with W CagA and inhibit its activity in 293T cells (Fig. 5*B*). In support, the inhibitory effect of W/AM-II was avoided when W CagA was forcibly premultimerized using AP20187, a small-molecule protein dimerizer (Fig. 5*B*). In a further illustration of AM-II CagA poisoning in mixed CagA multimers with implications for fitness, we found that W/AM-II CagA-expressing strains interfered with the ability of coinfecting W strains to achieve high titers in gerbil stomachs and to fully induce IKK-mediated IκB phosphorylation (Fig. 5, *C* and *D*).

DISCUSSION

Our study of geographically and genetically distinct types of *cagA* genes established that the CagA protein CRPIA motif is a key determinant of Hp virulence and fitness. We found that AM type CagAs, which are characteristic of Hp strains from Amerindians in the remote Peruvian Amazon village of Shimaa, were significantly attenuated in abilities to stimulate gastric epithelial proliferation and inflammation during infection.

A recent study indicated that such AM strains descend from Asian strains that arrived in the Americas with the ancestral Asian people some 15,000–20,000 years ago and that AM strains are less fit than and substantially displaced by hybrid or W strains in less isolated communities (27). Our *in vitro* and *in vivo* experiments indicated AM CagA proteins are less potent and contribute less to virulence and fitness than do W and EA CagA proteins. We, therefore, propose that AM *versus* W differences in CagA proteins contributed importantly to the apparent displacement of AM by W Hp strains in urban communities.

Intriguingly, residents of Satipo-region villages from elsewhere in the Peruvian Amazon watershed contained both AM and W strains, in contrast to only AM strains in Shimaa residents (Fig. 1*A* and supplemental Fig. S1). This might reflect

more contact with people from outside, *e.g.* during guerrilla wars of the late 20th century (37, 38). Continued studies of the genetics, virulence, fitness, and associated diseases of Hp from Amazonian and other Latin American populations promises to provide further insights into mechanisms of infection, persistence, and disease, especially in developing country at-risk human populations.

A mutational analysis of CRPIA motifs indicated that they could be key determinants of geographical differences in CagA-mediated host carcinogenic responses (Fig. 4*C* and supplemental Figs. S6–S8). Indeed, AM strains had less CagA activity than W and EA strains, with AM-II strains having markedly less than AM-I strains. Several nominally AM-II strains (Shi30, Shi35, Shi156) contained AM-I CRPIA motifs (Fig. 1*A*). These natural hybrids had activities similar to AM-I CagAs and higher than those of AM-II CagAs, as scored by induced Erk, Akt, and Met phosphorylation and IL-8 production (Fig. 2*B*). These results emphasize the importance of the CagA CRPIA motif in the development of Hp-related gastric illnesses.

Finally, we discovered that AM-II CagA can interfere with other CagA proteins both *in vitro* and *in vivo* (Fig. 5), an outcome suggesting new therapeutic approaches. Because current therapies for Hp infection are antibiotic-based and often compromised by antimicrobial resistance and also high rates of reinfection in developing country settings (39), new therapeutic strategies are needed to control Hp infections and diminish risks of associated gastric diseases (40). We propose considering AM-II strains such as Shi193 as novel live vaccines because these strains are highly human-adapted, well attenuated, and yet protective against infection by other more highly pathogenic strains.

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Clinical Microbiology

Analysis of outer membrane vesicle protein involved in biofilm formation of *Helicobacter pylori*

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ABSTRACT

Helicobacter pylori is one of the most common causes of bacterial infection in humans. Infection with *H. pylori* is closely associated with gastritis and peptic ulcers and is a risk factor for gastric cancer and mucosa-associated lymphoid tissue lymphoma. *H. pylori* forms biofilms on glass surfaces at the air–liquid interface in in-vitro batch cultures. We previously reported that strain TK1402 showed a strong biofilm-forming ability in vitro. We also suggested the outer membrane vesicles (OMV) produced by strain TK1402 might be related to its biofilm forming ability. In the present study, we analyzed the protein profile of the OMV produced by strain TK1402 and found a unique 22-kDa protein in TK1402 OMV cultured for 2–3 days. In addition, this protein could not be detected in the OMVs produced by other *H. pylori* strains. These results suggest that the 22-kDa protein is involved in effective biofilm formation by strain TK1402.

1. Introduction

Biofilms are ubiquitous in natural, industrial and clinical environments. They are surface bound communities of microbial cells found in oligotrophic environments and are strongly implicated in bacterial virulence [1]. Many bacteria in aquatic ecosystems have been demonstrated to exist in biofilms on a wide variety of surfaces. Among human bacterial pathogens, the biofilms of *Pseudomonas aeruginosa*, *Haemophilus influenzae*, pathogenic *Escherichia coli*, *Vibrio cholerae*, staphylococci and streptococci are some of the best studied [2–6]. Bacterial biofilms are frequently embedded in a self-produced extracellular matrix [7]. The extracellular polymeric substance (EPS) matrix, which can constitute up to 90% of the biofilm biomass, is a complex mixture of exopolysaccharides, proteins, DNA and other macromolecules [8].

Helicobacter pylori is a spiral, microaerophilic, noninvasive, Gram-negative bacterium that colonizes the human gastrointestinal tract, primarily the stomach [9] and previous studies have alluded to the ability of *H. pylori* to form biofilms [10,11]. A polysaccharide-containing biofilm was observed at the air–liquid interface when *H. pylori* was grown in a glass fermenter [10] and it was also capable of binding to a heterotrophic mixed species biofilm grown on stainless steel coupons [11]. We previously demonstrated that strain TK1402 exhibited strong biofilm formation [12]. This property might not be responsible for the cell hydrophobicity, aggregation, and motility in strain TK1402 [13]. We demonstrated that outer membrane vesicles (OMV) produced by strain TK1402 play an important role in the formation of the extracellular matrix of strain TK1402 biofilms [12]. OMV production is a physiologically normal function of gram-negative bacteria [14,15]. It has been also reported that the *H. pylori* strains released OMV into the extracellular space, and VacA, urease and lipopolysaccharides are present on the surface of OMV from *H. pylori* along with other outer membrane proteins [16,17]. However, which component(s) of the OMV contribute to biofilm formation remains to be determined. In the present study, we characterized the OMVs from strain TK1402.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The following *H. pylori* strains were used: SS1, ATCC 49503, ATCC 43579, NCTC11638, TK1029, TK1402, KR2003, and KR2005 (the last four are clinical isolates from Japanese patients). All strains were maintained at −80 °C in Brucella broth (Difco, Detroit, Mich) with

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20% (vol/vol) glycerol. These strains were cultured under microaerobic conditions at 37 °C on Brucella agar plate containing 7% horse serum (HS).

2.2. Isolation of outer membrane vesicle (OMV) structure

H. pylori strain TK1402 was grown on Brucella broth supplemented with 7% FCS for 1 day to 3 days. The cells were collected by centrifugation (10,000 g for 15 min), and the resulting supernatants were filtered (low protein binding Durapore membrane, 0.45 mm polyvinylidene fluoride, Millipore, Bedford, Mass.). The filtrates were centrifuged (40,000 g, 2 h at 4 °C), washed with PBS and re-centrifuged (40,000 g, 2 h at 4 °C). The pellets were next resuspended in PBS supplemented with 0.2 M NaCl. The OMV structures of other *H. pylori* strains cultured for 3 days in Brucella broth supplemented with 7% FCS were also isolated in a similar manner to that of strain TK1402.

2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The fractionated OMV from Brucella broth supplemented with 7% FCS in PBS were adjusted to an optical density of 2.0 at 280 nm. The adjusted OMV were treated with sodium dodecyl sulfate (SDS) loading buffer including 5% 2-mercaptoethanol at 100 °C for 5 min and separated by polyacrylamide gel electrophoresis (PAGE). The separated OMV proteins were stained with Coomassie brilliant blue (CBB).

2.4. Determination of N-terminal amino acid sequence

The OMV fraction was loaded onto gel and transferred to polyvinylidene difluoride membranes (Atto, Tokyo, Japan). After transfer, the membrane was stained with CBB. The band corresponding to approximately 22-kDa was excised from the gel. The N-terminal amino acid sequence was determined by ARPO science (Tokushima, Japan), based on the Edman degradation method.

3. Results

3.1. Evaluation of the OMV produced by TK1402

We previously demonstrated that strain TK1402 has a strong biofilm forming ability independent of its growth rate [12]. In addition, the OMV were located at the substratum–bacterium interface and extracellular space in the biofilm. We concluded that the OMV produced by strain TK1402 could play an important role for attachment to abiotic and cellular surfaces in the initial stages of biofilm formation.

Development of strain TK1402 from day 1 to day 6 demonstrated that the biofilm formation rapidly increased over 2 days and it took 3 days for biofilm maturation under these conditions. In order to examine the kinetics of OMV components before mature formation of the biofilm, the OMV were isolated from the cell cultures from day 1 to day 3 and characterized by SDS-PAGE (Fig. 1). The results indicated that the band profile of the OMV from day 1 displayed some remarkable differences compared to subsequent days. All OMV mostly shared similar bands, although the positions of the clear bands appear to be shifted downwards in the OMV from day 2 and day 3 compared to that from day 1. In addition, a 22-kDa band detected in the OMV from day 2 and day 3 was not detected in the OMV from day 1. There were no differences in the OMV between day 2 and day 3.

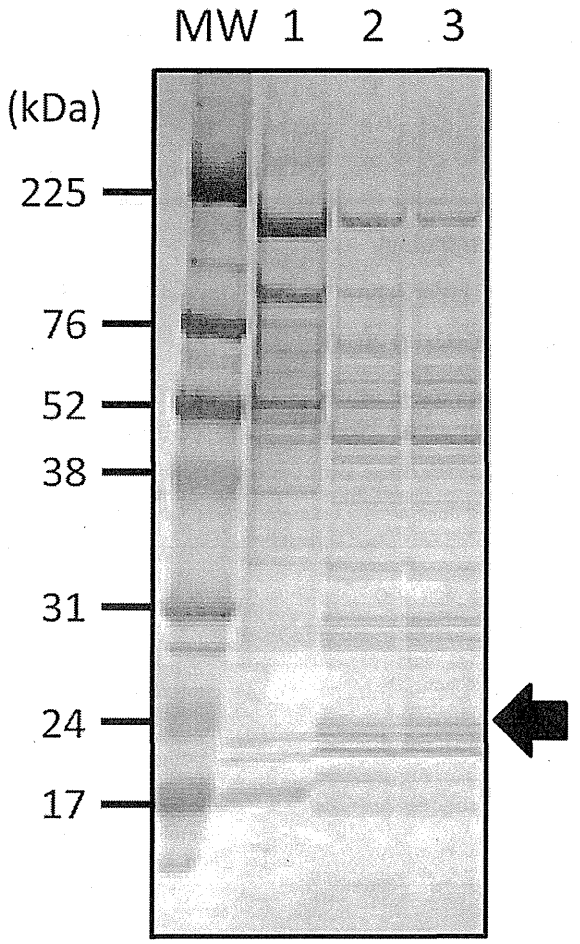

Fig. 1. The OMV were fractionated at day 1, 2 and 3 from TK1402 and the proteins were separated by SDS-PAGE (lane 1, 1-day culture; lane 2, 2-day culture; lane 3, 3-day culture). The approximate position of the 22-kDa band is shown by an arrow on the right side. Lane MW contains molecular weight markers. The data shown are representative of three independent experiments.

3.2. Differential OMV components in H. pylori strains

We attempted to compare the OMV components in the 8 strains of *H. pylori* with SDS-PAGE but all of the *H. pylori* strains except strain TK1402 formed very little biofilm biomass [12]. *H. pylori* strains were cultured for 3 days and the OMV were isolated. The band profile of the OMV seen by SDS-PAGE was variable but the 22-kDa band was detected only in the OMV from strain TK1402 (Fig. 2).

3.3. Amino acid sequences

Since the 22-kDa protein was only present in TK1402 OMV and this protein appeared before maturation of the TK1402 biofilm, we hypothesized that this protein might play an important role for biofilm formation in the strain. In order to characterize the 22-kDa protein, N-terminal amino acid sequencing was carried out. The five N-terminal residues that could be analyzed showed the amino acid sequence [VDFSK]. We attempted to search for a protein with a similar N-terminal sequence with Genbank but no other protein matched this N-terminus in the *H. pylori* database.

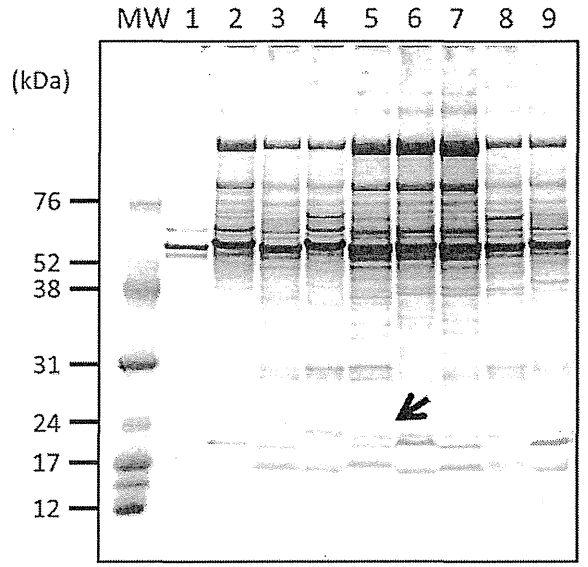

Fig. 2. The OMV were fractionated from 8 strains of *H. pylori* and the OMV were separated by SDS-PAGE (lane 1, medium control; lane 2, KR2003; lane 3, KR2005; lane 4, TK1029; lane 5, TK1402; lane 6, SS1; lane 7, NCTC11638; lane 8 ATCC 43503; lane 9, ATCC 43579). The approximate position of the 22-kDa band is shown by a black arrow. Lane MW contains molecular weight markers. The data shown are representative of three independent experiments.

4. Discussion

Recently, some of reports indicate that *H. pylori* has the ability to form biofilms on abiotic surfaces in-vitro as well as on the human gastric mucosa [11,18–21]. In our previous study, we demonstrated that strain TK1402, a clinical isolate derived from a Japanese peptic ulcer patient, has a strong biofilm forming ability [12] and that this property is associated with the production of OMV. In the present study, we analyzed the OMV with SDS-PAGE and found that the OMV from 1-day culture exhibited a different protein profile compared to those from 2-day and 3-day culture (Fig. 1). There were some bands present in the OMV from the 2- and 3-day cultures that were absent at day 1. This result indicated that the components of the OMV were different between 1 day culture and 2–3-day culture.

We further analyzed the OMV produced by 8 strains of *H. pylori* by SDS-PAGE with Coomassie blue staining and observed that there were several specific protein bands in the OMV from strain TK1402 which were absent in other strains of *H. pylori* (Fig. 2). It appears from our results that the clear 22-kDa band found in TK1402 OMV may be important in biofilm formation. The amino acid sequence determined by N-terminal sequencing was [VDFSK], but this amino acid sequence was not present in the *H. pylori* database. The reason for this may be that *H. pylori* strains exhibiting strong biofilm forming ability are relatively rare [12] and indeed a database strain, 26695, showed little biofilm formation (data not shown). These data suggest that 22-kDa protein may play a part of important role in biofilm formation of strain TK1402. However, the role of 22-kDa protein for biofilm formation still remains to be determined. Identification, purification, and chemical characterization will be required to clarify the 22-kDa protein. Additional investigations are now in progress to analyze it. Biofilm formation is critical for not only environmental survival but also successful infection in numerous pathogenic bacteria. Our present results provide useful information for understanding of biological significance of *H. pylori* biofilm formation.

5. Conclusions

We have demonstrated that strain TK1402 has a strong biofilm forming ability. In addition, the results suggested that this property might be dependent upon a 22-kDa protein obtained from the OMV of this strain. This may represent a potentially novel gastric cell colonization factor for this organism.

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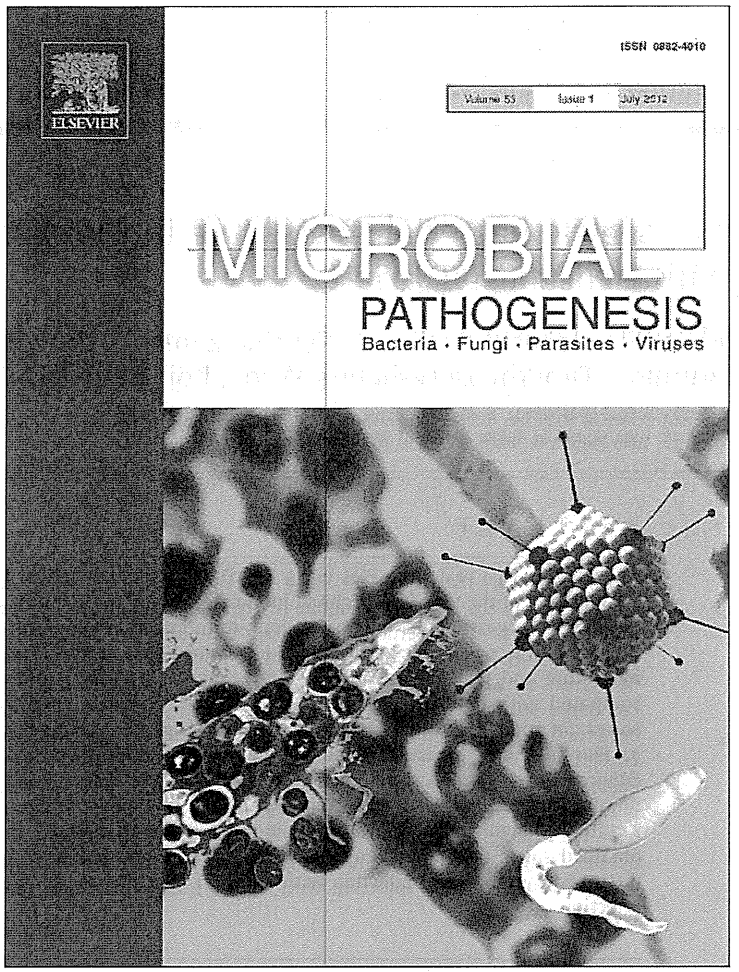

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Comparative analysis of gastric bacterial microbiota in Mongolian gerbils after long-term infection with *Helicobacter pylori*

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ABSTRACT

Quantitative (qt) real time PCR using 16SrDNA primers is useful for determination of the bacterial composition of the gastric microbiota in Mongolian gerbils. The aim of this study was to determine the change in the gastric microbiota after long-term infection with *Helicobacter pylori*. One year after inoculation with *H. pylori*, five gerbils were determined as *H. pylori*-positive and 6 gerbils *H. pylori*-negative by culture and real time qt PCR methods. The gastric microbiota of each group of gerbils was also compared with that of 6 gerbils uninfected with *H. pylori*. DNA from the *Atopobium* cluster, *Bifidobacterium* spp., *Clostridium coccoides* group, *Clostridium leptum* subgroup, *Enterococcus* spp. and *Lactobacillus* spp. were detected in the gastric mucus of both infected and uninfected gerbils. In contrast, *Eubacterium cylindroides* group and *Prevotella* spp. were detected only in *H. pylori*-negative gerbils. The numbers of *C. leptum* subgroup, *C. coccoides* group and *Bifidobacterium* spp. in gastric mucus of *H. pylori*-negative Mongolian gerbils were significantly lower than those in non-infected gerbils. The results obtained suggest that the composition of gastric indigenous microbiota in Mongolian gerbils may be disturbed by long-term infection with *H. pylori*, and that these changes may in fact inhibit *H. pylori* infection.

1. Introduction

Helicobacter pylori infection of the human gastric mucosa induces gastritis [1,2] and is related to many gastric diseases including gastric cancer [3]. Infection continues throughout the life of the patient and influences not only gastroduodenal diseases but also gastric environment and function [4]. In our previous study, persistent infection of the gastric mucosa of Mongolian gerbils with *H. pylori* could be detected by real-time reverse transcription (RT)-PCR for 6 months after infection, but *H. pylori* could not be isolated by culture methods after 4 months [5]. Also, it was noted that the change in the gastric environment caused by *H. pylori* infection may affect not only the number of *H. pylori* bacteria, but also the indigenous gastric bacterial composition.

Identification of bacterial strains by conventional culture-based methods provides an incomplete and biased picture of the biodiversity of intestinal microbiota, as many species cannot be cultivated *in vitro* [6]. Therefore, culture-independent molecular methods based on 16S rRNA genes such as fluorescent *in situ* hybridization [7,8], dot-blot hybridization with rRNA-targeted probes [9], denaturing gradient gel electrophoresis [10,11], temperature gradient gel electrophoresis [12], and cloning and sequencing of rDNA [6,13] have been introduced to obtain a better understanding of the gut microbiota. Recently, Matsuki et al. [14–16] reported a quantitative PCR method with 16S rRNA-gene-targeted species-specific primers to species including *Bacteroides fragilis* group, *Bifidobacterium*, *Clostridium coccoides* group and *Prevotella*, which were designed for analysis of human intestinal microbiota.

H. pylori is inhibited by a number of commensal bacterial species as well as opportunistic human pathogens [17]. The aim of the present study was to determine the differences in the gastric microbiota between *H. pylori*-positive and negative gerbils. We determined the composition of the gastric microbiota of Mongolian gerbils after long-term infection with *H. pylori* and compared infected and non-infected gerbils to elucidate any increasing or

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